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USE OF LEUCOCYTE METABOLISM AS A HEALTH EFFECTS INDICATOR

Kenneth D. Lunan

Stanford Research Institute

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Rabbit leucocytes were also treated *in vitro* with sodium hexachloroplatinate, and the same metabolic capabilities were assessed. Lead and cadmium treatments produced a mild anemia, but the white cells were only slightly affected. The one-week cadmium treatment and the three-week lead and cadmium treatments depressed the synthesis of both nucleic acids. The synthesis and degradation of protein and phospholipid were unaffected by the metal treatments. Leucocytes from three-week control rabbits synthesized all four classes of biomolecules at a faster rate than leucocytes from the one-week control rabbits. In leucocytes treated with the platinum salt *in vitro*, nucleic acid and protein synthesis were depressed, but phospholipid synthesis was unaffected.

These results demonstrate that leucocytes may serve as a responsive bioindicator of trace metal contamination.

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Rabbit leucocytes were also treated *in vitro* with sodium hexachloroplatinate, and the same metabolic capabilities were assessed. Lead and cadmium treatments produced a mild anemia, but the white cells were only slightly affected. The one-week cadmium treatment and the three-week lead and cadmium treatments depressed the synthesis of both nucleic acids. The synthesis and degradation of protein and phospholipid were unaffected by the metal treatments. Leucocytes from three-week control rabbits synthesized all four classes of biomolecules at a faster rate than leucocytes from the one-week control rabbits. In leucocytes treated with the platinum salt *in vitro*, nucleic acid and protein synthesis were depressed, but phospholipid synthesis was unaffected.

These results demonstrate that leucocytes may serve as a responsive bioindicator of trace metal contamination.

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INTRODUCTION

Of increasing concern are the public health hazards associated with the insidious accumulation of heavy metals in the bodies of man and animals as a result of contamination of the environment with an increasing amount and variety of trace metals derived from industrial sources. Any excess of environmentally derived material beyond that needed for optimun growth and development may be termed a "pollutant burden." It can be assumed that any biological response resulting from a pollutant burden is undesirable. In a practical sense, it may be sufficient to limit a pollutant burden to that level that produces a biological response with uncertain physiological significance. Higher levels that may herald incipient disease would be regarded as intolerable.

The problem of recognizing the level of a pollutant burden at a preclinical stage in an individual is formidable. A preclinical biological response induced by the pollutant burden may manifest itself as a metabolic or physiologic alteration. The use of leucocytes as a bioindicator offers considerable advantages not available in other readily accessible biological materials. The most important feature is that the leucocyte is a complete cell that functions in all major areas of metabolism. By examining the response of the leucocyte to a test reagent, it may be possible to assess the metabolic status of the individual. Numerous examples exist in which the leucocytes from individuals suffering from inborn errors of metabolism exhibit the same metabolic characteristics as their host. Also, because it is derived from the reticuloendothelial system and therefore possesses immunological characteristics not found in other cells and because of its broad range of body functions, the leucocyte undoubtedly will find wider use as a bioindicator in many areas of environmental research.

The research described in this report was undertaken to assess the use of leucocytes as a bioindicator of trace metal contamination using lead and cadmium. In the event that one or more measurable metabolic processes in leucocytes are effected by heavy-metal treatment of mammals, then these results may be applicable to the determination of damage in man due to chronic exposure to heavy metals.

A large portion of the work on this contract was devoted to developing appropriate methods for the isolation and culture of leucocytes in the rabbit. Although rats were originally proposed as the experimental animal, rabbits were finally chosen by mutual agreement between EPA and SRI to facilitate the isolation of larger quantities of leucocytes than would be possible from the rat. The methods found in the literature for leucocyte preparation and culture are largely devoted to human cells, and it soon became apparent that these methods were not adequate for animal

cells. Late in the contract year, Mansfield and Wallace described solutions for many of the difficulties encountered in this research. This led to modifications resulting in a successful method for the preparation and culture of rabbit leucocytes. The principal difference in methodology is the inclusion in the culture medium of autologous rabbit plasma as the blood protein supplement.

The main body of results reported herein concerns the experiments in which animals were injected daily for one or three weeks with physiological saline, lead acetate, or cadmium chloride solutions. Some preliminary results with control animals and with those treated for two weeks are also reported.

The final experiment deals with the in vitro effect of sodium hexachloroplatinate on rabbit leucocytes. This form of platinum was chosen by mutual agreement between EPA and SRI as the third metal to be studied in this research.

METHODS AND RESULTS

Preliminary Experiments

Biochemical Assays

Before leucocytes became available to us, lung tissue was used to perfect various biochemical assays. Lipids were extracted with chloroform/methanol (2:1) and weighed [1]. Acid-soluble nucleotides were removed in ice-cold 0.2% perchloric acid. RNA was removed by digesting the residue for four hours at 37°C in 1 N KOH. DNA was subsequently removed by digesting for one hour at 65°C in 10% perchloric acid. These materials were determined spectrophotometrically [2]. The residual protein was dissolved in base and determined by the Lowry method [3].

The data in Tables 1 and 2 represent typical analyses of lung tissue. Table 1 shows that longer alkaline hydrolysis yields slightly more RNA and less DNA, with the total nucleic acids remaining about the same. These data attest to the validity of the observations of Fleck and Munro [4] that the efficiencies of the alkaline hydrolysis for RNA and of the acid hydrolysis for DNA depend on the tissue being analyzed. Further, the separation of DNA and protein is a balance between maximizing DNA extraction and minimizing protein degradation [4]. Thus, the hydrolysis conditions employed here for lung tissue may not be optimal for leucocyte analysis. Table 2 shows the reproducibility of these assays. The lack of agreement between the two tables is due to the differences in age of the lung tissue.

Table 3 presents data for one leucocyte preparation.

Toxicity Studies

Intraperitoneal Toxicity (LD_{50})

Adult New Zealand white rabbits of mixed sex, each weighing 1.87 to 2.97 kg, were used. Lead and cadmium salts were administered intraperitioneally in graded dosages. Tables 4 and 5 summarize the intraperitoneal toxicity (LD_{50}) of lead and cadmium, respectively, calculated by the methods of Thompson and Weil [5] and Weil [6].

Lead--The source of lead was lead acetate (Baker, AR Grade), expressed as $Pb(CH_3COO)_2 \cdot 3\,H_2O$. Water was the diluting vehicle used in administering the compound, as a 50% (w/v) aqueous solution.

Table 1

VARIATION IN RNA AND DNA FROM REPLICATE SAMPLES OF RAT LUNG EXTRACTED WITH ALKALI FOR ONE AND FOUR HOURS[a]

Experiment	Conditions	Acid-Soluble Nucleotides	RNA	DNA	Total Nucleic Acids
1	1.0 N NaOH, 1 hr	1.49	2.51	6.40	8.91
		1.42	2.80	6.47	9.27
		Avg 1.46	2.66	6.44	9.09
2	1.0 N KOH, 4 hr	1.44	3.24	5.31	8.55
		1.43	4.03	5.35	9.38
		Avg 1.44	3.64	5.33	8.96

[a] Units in mg constituent per gram wet tissue.

Table 2

REPLICATE ANALYSIS OF RAT LUNG TISSUE

Sample No.	Lipids	Acid-Soluble Nucleotides	RNA	DNA	Total Nucleic Acids
			Wet Tissue Weights[a]		
1	88.5	0.433	3.17	2.63	5.80
2	88.7	0.406	3.34	2.61	5.95
3	89.1	0.388	3.25	2.40	5.65
4	96.3	0.352	3.18	2.60	5.78
5	99.4	0.326	3.19	2.64	5.83
6	96.0	0.385	3.13	2.40	5.53
Mean	93.0	0.382	3.21	2.55	5.76
Std. Dev.	± 4.79	± 0.0380	± 0.0753	± 0.112	± 0.147
Std. Error	± 1.96	± 0.0166	± 0.0307	± 0.0456	± 0.0599
			Dry Tissue Weights[b]		
1	532	1.70	12.4	10.3	22.7
2	527	1.58	13.1	10.1	23.2
3	539	1.52	12.8	9.43	22.2
4	570	1.33	12.0	9.80	21.8
5	588	1.21	11.9	9.81	21.7
6	570	1.46	11.8	9.10	20.9
Mean	554	1.47	12.3	9.77	22.1
Std. Dev.	± 24.9	± 0.175	± 0.515	± 0.448	± 0.808
Std. Error	± 10.2	± 0.0714	± 0.210	± 0.183	± 0.330

[a] Units in mg constituent per gram wet tissue.

[b] Units in mg constituent per gram dry tissue.

Table 3

PRELIMINARY ANALYSIS OF RAT LEUCOCYTES[a]

Sample	Lipids	Acid-Soluble Nucleotides	RNA	DNA	Total Nucleic Acids
1	58.0	0.0703	0.762	5.83	6.59

[a]Units in mg constituent per gram wet tissue.

Levels of 79, 178, 400, 1350 and 4560 mg/kg were used in the rangefinding study. Based on these preliminary dosages, subsequent treatment levels of 178, 267, 400, 600, 900 and 1350 mg/kg were used to determine the single dose LD_{50} for lead, as lead acetate. Restlessness and rapid breathing, lasting from 10 minutes to three hours, occurred as the dose increased from 600 to 1350 mg/kg. At time of sacrifice or death, blood-tinged fluid was found in the peritoneal cavity at these dosages. At lower doses of 178 to 400 mg/kg, the rabbits appeared restless and exhibited rapid breathing immediately after injection and for the next 15 to 40 minutes; necropsy of all animals revealed no gross pathological changes.

The LD_{50} of lead acetate is 470 mg/kg, with 95% confidence limits of 283 to 780 mg/kg.

Cadmium--The source of cadmium was cadmium chloride (Mallinckrodt, AR grade), expressed as $CdCl_2.2\text{-}1/2H_2O$. The compound was administered as an aqueous solution at varying concentrations in a constant volume of 1 ml/kg.

Levels of 35.1, 52.7, 79, 118, 178, and 267 mg/kg were used in the range-finding study. Based on these preliminary dosages, subsequent treatment levels of 3.08, 4.62, 6.93, 10.4, 15.6, and 23.4 mg/kg were used to determine the single dose LD_{50} for cadmium, as cadmium chloride. Restlessness and rapid breathing were observed at all levels, with increasing severity and duration of effects as the dosage increased. Necropsy of all animals showed the presence of blood-tinged fluid in the peritoneal cavity.

The LD_{50} of cadmium chloride is 8.5 mg/kg, with 95% confidence limits of 2.46 to 29.3 mg/kg.

Table 4

I.P. LD_{50} DATA FOR RABBITS TREATED WITH LEAD ACETATE

Dose (Mg/Kg)	Number Dead/ Number Treated	Time to Death	I.P. LD_{50} (Mg/Kg)
178	0/4♂	--	
267	2/6♂	3-1/2 - 19 hrs	
400	2/5♂	3-1/4 - 18 hrs	470
600	4/4♂	1 - 5-1/2 hrs	(283 - 780)[a]
900	3/4♂	4 hrs - 3 days	
1350	8/9♂	2-3/4 hr - 6 days	

[a]95% confidence limits.

Table 5

I.P. LD_{50} DATA FOR RABBITS TREATED WITH CADMIUM CHLORIDE

Dose (Mg/Kg	Number Dead/ Number Treated	Time to Death	I.P. LD_{50} (Mg/Kg)
3.08	0/4♂	--	
4.62	2/4♂	2 - 8 days	
6.93	2/4♀	2 - 4 days	8.50
10.4	2/4-1♂,3♀	18-1/2 hr - 2 days	(2.46 - 29.3)[a]
15.6	1/4♂	20 hr	
23.4	4/4-1♂,3♀	2 hr - 1 day	

[a]95% confidence limits.

Repeated Intraperitoneal Injections

Adult New Zealand white rabbits, each weighing 2.35 to 2.97 kg, were used. Three rabbits per treatment level received 21 repeated injections 24 hours apart. Table 6 summarizes the toxicity data for both lead and cadmium. Levels of 75 and 25 mg/kg for lead acetate and 1 mg/kg for cadmium chloride were used.

The three rabbits that received 75 mg/kg lead acetate showed diarrhea and emaciation after five days of treatment, with weight losses in excess of 25%. Treatment was terminated after five days, but the rabbits were held to observe post-treatment effects. Recovery from treatment did not occur; the three rabbits died at two, three, and seven days post-treatment.

At 25 mg/kg, one rabbit died after ten injections and another after 15 injections. The third rabbit completed the 21 injection regimen, but died six days post-treatment. Body weight losses were 20, 37, and 28%, respectively, for the three rabbits. All three rabbits treated with 1 mg/kg cadmium chloride survived the 21-day exposure period, body weight losses were 15% or less.

After evaluation of the data on repeated intraperitioneal injections, 10 mg/kg for lead acetate and 1 mg/kg for cadmium chloride were selected as the treatment levels for the one-week and three-weeks experiments.

Preparation of Leucocytes

In these and all subsequent experiments, rabbit blood was obtained by cardiac puncture (see the section "The Procedure of Mansfield and Wallace," pg. 28).

Isolation Experiments Using Ficoll-Hypaque

A suspension of purified, erythrocyte-free leucoytes was prepared by a one-step, density gradient centrifugation, using Ficoll and Hypaque* as the gradient material [7,8]. Heparinized rat blood diluted 1:1 with saline was layered over the gradient and centrifuged. The leucocytes layered on top of the gradient, while the erythrocytes sedimented to the bottom of the tube below the gradient and completely separated

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*Hypaque is the trade name for sodium 3,5-diacetamide-2,4,6-triiodobenzoate, also called sodium diatriazoate (Winthrop Laboratories). Most literature references refer to the use of Isopaque, also a product of Winthrop, as a gradient material. However, Isopaque, the N-methyl derivative of Hypaque, is no longer commercially available because of its toxicity.

Table 6

SUMMARY DATA FOR REPEATED INTRAPERITONEAL TOXICITY OF LEAD ACETATE AND CADMIUM CHLORIDE

Dose (Mg/Kg)	Rabbit No. and Sex	Body Wt (kg) 0	1 Wk	2 Wk	3 Wk	% Body Wt. Loss	Time to Death
			Lead Acetate				
75[a]	237 M	2.73	2.05	--	--	25	7 days
	238 M	2.66	1.78	--	--	33	8 days
	239 M	2.35	1.72	--	--	27	12 days
25	243 F	2.29	1.70	1.44	--	37	15 days
	244 F	2.74	2.44	2.42	1.96	28	27 days
	245 F	2.97	2.37	--	--	20	10 days
			Cadmium Chloride				
1	240 F	2.96	2.63	2.56	2.53	15	--
	241 F	2.70	2.35	2.60	2.52	7	--
	242 F	2.91	2.54	2.27	2.48	15	--

[a]Treatment terminated after 5 days--rabbits severely dehydrated, with body weight loss.

from the leucocytes. The bulk of the plasma layer above the leucocytes was aspirated, and the leucocytes were removed with a Pasteur pipette, washed with Hank's balanced salts solution (HBSS), and resuspended in HBSS. Approximately 0.8 x 10^6 leucocytes/ml of whole blood was recovered. Although cell recovery was not determined in this experiment based on a typical WBC of 5 x 10^6 leucocytes/ml of whole blood, the recovery can be estimated at approximately 16%. The Trypan blue dye exclusion test for cell viability indicated over 95% viability.

Rat leucocytes were prepared and incubated overnight at a concentration of 2 x 10^6 cells/ml in Eagle's minimal medium (MEM) (containing 20% calf serum, 2 mM glutamine, and 250 units/ml each of penicillin and streptomycin) at 37°C.* Difco phytohemagglutinin-M (PHA-M) (0.05 ml/2 ml of cell suspension) was added, and the cultures were incubated for four days. At the end of four days, the cell count, initially 2 x 10^6/ml, had risen slightly and viability remained at about 97% of total cells.

The cultures then received 1 μc/ml of each of the following radioactive substrates: 5-^{3}H-uridine, 2-^{14}C-thymidine, ^{3}H-ℓ-leucine (U), and 1,2-^{14}C-choline chloride. After incubation of the tubes for two hours, the cells were harvested and washed with medium containing a 100-fold excess of the above unlabeled compounds. Lipid, acid-solubles, RNA, DNA, and protein were then extracted from one portion of the cells and assayed for radioactivity in a liquid scintillation spectrometer. The remaining cells were resuspended in more medium containing the unlabeled compounds and were incubated an additional two hours. The cells were then harvested, washed, and extracted in the same way as the first group of cells.

The data in Table 7 follow the expected pattern, with the cells from Tubes 2 and 4 (representing a two-hour degradation period) having a significantly lower level of counts than the cells from Tubes 1 and 3. Thus, at the end of two hours, lipid had declined to 3% of the zero-time value. Similarly, RNA, DNA, and protein declined to 12%, 18%, and 22%, respectively. Therefore, these data indicate a significant rate of degradation of lipid, protein, and nucleic acid.

The major difficulty encountered in this experiment was the small amount of cellular material and, consequently, the small amounts of lipid, RNA, DNA, and protein extractable from the cells.

This initial success in both the preparation and culture of leucocytes was found difficult to reproduce. One problem that became readily apparent was due to the pH of the medium. When the gas phase above the medium was replaced with 5% CO_2 in air at the time of inoculation, the medium retained the proper pH throughout the culture period. Nevertheless, the practice was adopted of renewing the gas phase daily. The deleterious effect of not controlling the pH is indicated by the

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*The culture conditions employed were a synthesis of the best features described in References 9-12.

Table 7

BIOSYNTHESIS AND DEGRADATION OF LIPIDS, NUCLEIC ACIDS, AND PROTEIN IN CONTROL RAT LEUCOCYTES

	Lipid	Acid-Solubles	RNA	DNA	Protein
Radioactive substrate:	Choline	--	Uridine	Thymidine	Leucine
Label:	C^{14}	H^3 or C^{14}	H^3	C^{14}	H^3
Measurement:	DPM-C^{14}	DPM-C^{14}	DPM-H^3	DPM-C^{14}	DPM-H^3

Tube	Post-Incubation	Serum	Lipid	Acid-Solubles[a]	RNA	DNA	Protein
1	0	Fetal	420[b]	1074	95.6	286	52.8
2	2 hr	Fetal	14.1[c]	200	22.4	0	51.3
3	0	Calf		1383	86.1	422	45.3
4	2 hr	Calf		220	0	131	0

[a]When counted for H^3, Tube 3 gave a value of 49.72 disintegrations per minute (DPM); the others were zero.

[b]Combined counts for Tubes 1 and 3.

[c]Combined counts for Tubes 2 and 4.

data in Table 8. Although sterile conditions were not strictly enforced in preparing these preliminary cultures, bacterial contamination was not evident in any of these culture experiments, thus attesting to the efficacy of the antibiotics included in the culture medium.

Rat and rabbit leucocytes obtained by the density gradient centrifugation method developed for human blood [7,8,13] were consistently low in white cell count and had a high proportion of residual erythrocytes.

Considerable effort was devoted to improving the leucocyte yield and lowering the residual erythrocyte count prior to the development by Mansfield and Wallace [14] of their improved technique, which was adopted in this research. Although it appears that the erythrocytes cannot be completely eliminated, acceptable yields of white cells have been attained.

Initially separation experiments were performed in plastic disposable centrifuge tubes until it was observed that white cells apparently adhered to the walls of these tubes. Table 9 shows the low recovery of white cells in these experiments. In all succeeding experiments, glass tubes were used, and recoveries were generally improved. The leucocyte count varied with the individual animals but averaged about 5×10^6/ml of whole rabbit blood.

Table 10 shows the improved recoveries of leucocytes from rabbit blood when glass tubes were used and when the centrifugation step was eliminated. Higher recovery is favored by separation at 37°C, but longer separation time, dilution of the blood, application of blood to the gradient, and increase of the osmolarity of the gradient are all somewhat deleterious to high white cell counts. These factors also raise the proportion of red cells in the white cell suspension. Increased temperature tended to reduce red cell count. Elimination of the centrifugation step increased the proportion of red cells nearly 20-fold; at the same time, the white cell count was doubled (Table 11).

Table 11 also shows the improved white cell yield obtained when the density of the gradient was reduced. (The density is directly related to the relative proportion of Hypaque; thus, when the proportion of Ficoll is increased, the density decreases.) However, even though the red cell count is diminished at lower densities, it is still too high.

Previously, hypotonic lysis has been employed to eliminate contaminating erythrocytes from human leucocyte suspensions, evidently without adverse effects on the white cells, as judged by electron microscopy, viability, and several biochemical parameters [15]. In one case, rabbit white cells were subsequently cultured for 18 hours [16].

Hypotonic lysis [15] was employed to reduce the red cell count, as illustrated in Table 12. Although these data are not included in this table, the red cell count averaged less than one red cell per white cell. Thus, the lysis procedure was markedly effective in reducing the number of red cells but not in eliminating them. However, comparison of the data from Tables 11 and 12 reveals that the lysis also reduces the white

Table 8

VIABILITY OF CULTURED LEUCOCYTES

	Without CO_2				5% CO_2			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
Day	Cell Count	Viability (%)	Cell Count	Viability (%)	Cell Count	Viability (%)	Cell Count	Viability (%)
0	1.70	97	1.12	95	2.2	96	2.0	94
			1.15	94				
1	0.376		0.08	40	1.7	97		
	0.472		0.048	33				
2	0.304	63						
	0.336	68						
3	0.226	7						
	0.304							
4							0.78	69

Table 9

RECOVERIES OF LEUCOCYTES FROM RAT AND RABBIT BLOOD IN PLASTIC CENTRIFUGE TUBES[a]

Experiment	Blood Sample	WBC[b]	Viability (%)	RBC/WBC
1	Rabbit	0.072	>95	
2	Rabbit	Low		
3	Rabbit	0.10		0.66
4	Rat	0.42	73	

[a] Whole blood was diluted with four volumes of saline and layered onto five volumes of Ficoll/Hypaque solution [9% (w/v) Ficoll in water, 24 volumes, and 34% (w/v) Hypaque in water, 10 volumes]. After centrifuging at 700–1700 x g for 30 min, the interfacial band containing leucocytes was drawn off. The leucocytes were washed in saline three times.

[b] WBC recovered per ml of whole blood, x 10^{-6}.

Table 10

RECOVERY OF LEUCOCYTES FROM RABBIT BLOOD[a]

Experiment	Gradient	Temperature (°C)	Time (min)	Blood	WBC[b]	RBC / WBC
1	Standard	20	60	Whole	1.76	24.4
2	Standard	37	60	Whole	2.30	21.3
3	Standard	5	60	Whole	1.82	26.3
4	Standard	20	240	Whole	0.80	40.0
5	Standard	20	60	c	0.35	125
6	Standard	20	60	Whole[d]	1.49	34.5
7	Standard[e]	20	60	Whole	1.39	40.0

[a]Separation performed as in Table 9, except that whole blood was layered onto gradient and, instead of centrifugation, the samples were allowed to stand for the specified time.

[b]WBC recovered per ml of whole blood, x 10^{-6}.

[c]One volume of blood/saline (1:1) was layered onto the gradient.

[d]Two volumes of whole blood were layered onto the gradient.

[e]Ficoll/Hypaque made up in 1% saline.

Table 11

RECOVERY OF RABBIT LEUCOCYTES
IN VARIOUS DENSITY GRADIENTS

Ficoll: Hypaque[c]	Experiment 1[a] WBC[d]	Experiment 1[a] RBC/WBC	Experiment 2[b] WBC[d]	Experiment 2[b] RBC/WBC
20:10			1.26	25.0
24:10[e]			1.76	24.4
26:10	0.93	1.94		
28:10			2.19	21.3
30:10	1.08	1.04		

[a]Performed as in Table 9; centrifuged at 700 x g.

[b]Performed as in Table 10; no centrifugation.

[c]Gradient composition as in Table 9, except proportions are varied.

[d]WBC recovered per ml whole blood, x 10^{-6}.

[e]Standard gradient used in Tables 9 and 10.

cell count by a factor ranging from 5 to 20. Another disturbing observation is that vital staining revealed possible damage to the nuclear membrane as a result of the lysis step. After lysis, nuclear material was observed throughout the cell interior, whereas before lysis, a clear nuclear structure could be observed.

Rat blood gave markedly higher white cell counts following hypotonic lysis than did rabbit blood, as shown in Table 13. Increasing the time of separation, the temperature of separation, or the time of hypotonic lysis, as well as including low-speed centrifugation following separation, significantly reduced the subsequent white cell count. In all cases, the white cells appeared damaged, as judged by vital staining.

Other attempts to lower the red cell count were unsuccessful. A second separation on Ficoll-Hypaque before hypotonic lysis and PHA-M treatment prior to separation was not effective in either heparinized blood or in EDTA-treated blood. PHA-M is a red cell agglutinating agent as well as a mitogen.

Table 12

RECOVERY OF RABBIT LEUCOCYTES FOLLOWING HYPOTONIC LYSIS[a]

Experiment	Time for: Separation (min)	Time for: Hemolysis (sec)	Centrifugation After Separation	WBC[b]
1	30	30	No	0.13
2	60	30	No	0.43
3	90	30	No	0.21
4	120	30	No	0.45
5	30	15	No	0.35
6	30	30	No	0.13
7	30	60	No	0.29
8	30	90	No	0.08
9	30	30	No	0.13
10	30	30	100 rpm	0.15
11	30	30	200 rpm	0.03, 0.19
12	30	30	400 rpm	0.93
13[c]	30	30	No	0.48
14[d]	30	30	No	0.48

[a] The procedure is the same as that in Table 10, except that the separation time was 30 min at 5°C. The plasma layer was removed and centrifuged. The plasma was decanted, and the cells were resuspended in 2 ml of saline. Hypotonic lysis was effected by adding 6 ml of water and mixing by inversion for 30 sec. Then 2 ml of 3.5% saline was added. The cells were sedimented and washed three times in saline.

[b] WBC recovered per ml of whole blood, x 10^{-6}.

[c] After removal of the plasma, the cells were resuspended in saline containing 0.06% EDTA.

[d] Whole blood was mixed with 0.2 volumes of saline and 0.02 volumes of PHA-M.

Table 13

LEUCOCYTE RECOVERY FROM RAT BLOOD FOLLOWING HYPOTONIC LYSIS[a]

Experiment	Time for: Separation (min)	Time for: Hemolysis (sec)	Centrifugation After Separation	Separation Temperature (°C)	WBC[b]	RBC / WBC
1	60	30	No	25	1.3	
2	60	30	No	5	2.5 3.0	
3	60	30	100 rpm	5	2.2 2.2	
4	60	30	200 rpm	5	1.1 1.9	
5	60	30	No	5	1.7 1.7	0.71 0.35
6	60	60	No	5	1.2 1.1	2.6 1.8
7	90	30	No	5	1.0 1.3	2.8 2.4
8[c]	60	30	No	5	1.7 1.3	1.6 0.65

[a]The procedure is the same as that in Table 12, except that the separation time was 60 min.

[b]WBC recovered per ml of whole blood, x 10^{-6}.

[c]Whole blood was pretreated with PHA-M as in Table 12, footnote d.

Isolation Experiments Using Gelatin

The second procedure tried was the older method of using gelatin solutions as a settling medium [17]. The general procedure is as follows. All glassware is sterile and silicon-treated. A fresh solution of 3% gelatin in 0.85% saline is prepared and maintained at 45°C until it is used; gelatin solutions over 2 hours old are not used. Blood is collected in EDTA (or is defibrinated), mixed with gelatin (3:1), and incubated at 37°C for 30 to 90 minutes. The clear supernatant containing the leucocytes is centrifuged for 10 minutes at 1500 x g and washed with an equal volume of HBSS. The cells are resuspended in MEM-S culture medium containing 25 mM HEPES and incubated at 37°C. The following experiments were conducted using this procedure except where noted.

Experiment 1. In this initial experiment, rat or rabbit blood was used with varying concentrations of gelatin and times of separation. As shown in Table 14, this resulted in a high yield of white cells and low red cell counts. Longer separation times in the gelatin, diluted blood, and defibrinated blood diminish the recovery of white cells. The latter two procedures also produce high red cell counts in the final suspension. Gelatin at 3.5% appears to give the best yield of white cells.

Experiment 2. Rabbit or human blood in EDTA or heparin was separated in silicon-treated und untreated glassware. As judged by the relative clearness of the supernatant layer after separation, the rabbit blood in EDTA separated in silicon-treated glassware appeared to give the best removal of RBC. The rabbit blood seemed to be as well separated as the human blood. White cells from untreated glassware were observed to clump.

Experiment 3. White blood cells were isolated from defibrinated or EDTA-treated rabbit blood and then cultured. After 24 hours, the leucocytes had settled out of the medium. The cells isolated from EDTA blood were difficult to resuspend and showed fibrin-like strands. The cells isolated from defibrinated blood resuspended easily and did not form strands. The cells were examined under a microscope but were not counted. The RBC contamination appeared high (RBC/WBC $\approx$ 5 to 10), and WBC viability appeared low (below 20%), as judged by the Trypan blue test.

Experiment 4 (incubation time). We attempted to improve RBC removal by varying the incubation time of the defibrinated blood-gelatin mixture between 60 and 120 minutes. The best separation time was found to be 90 minutes.

Experiment 5. WBC were isolated from 4 samples of defibrinated rabbit blood at a separation time of 90 minutes. Counts were made immediately following isolation. The following tabulation presents the results:

Table 14

RECOVERY OF RAT AND RABBIT LEUCOCYTES AFTER SEDIMENTATION IN GELATIN[a]

Experiment	Blood	Gelatin (%)	Time of Separation (min)	WBC[b]	Percentage of WBC Recovery	RBC/WBC
1	Rat	--	60[c]	0.76	29	0.19
				0.78	29	0.19
2	Rat	3.0	90	0.48	18	4.3
3	Rat	2.5	90	0.93	35	2.3
				0.67	25	2.3
4	Rabbit	3.0	60	7.59	61	2.03
5	Rabbit	3.0	90	5.83	47	1.43
6	Rabbit	3.0	120	5.05	41	1.60
7	Rabbit	2.5	90	7.75	62	1.12
8	Rabbit	3.5	90	9.66	78	1.35
9	Rabbit	3.0[d]	90	4.11	33	9.05
10	Rabbit	3.0[e]	90	3.23	47	10.2

[a] One volume of EDTA-treated whole blood is mixed with 0.5 volume of 3% gelatin in saline and allowed to stand for 90 min at 37°C. The clear upper layer is removed and the white cells are isolated by centrifugation. The cells are washed twice in saline and resuspended in culture medium at a concentration of 1 x 10^6 cells/ml.

[b] WBC recovered per ml whole blood, x 10^{-6}.

[c] White cells were isolated as described in Table 8, with a 60-min separation followed by hypotonic lysis for 20 sec.

[d] One volume of whole blood diluted 1:1 with saline mixed as in footnote a.

[e] Defibrinated whole blood mixed as in footnote a.

Sample	WBC[a]	% Viable WBC[b]	RBC/WBC
1	1.36	73	4.3
2	1.09	74	3.4
3	1.40	70	3.9
4	1.40	63	4.3

[a]WBC (10^{-6}) recovered/ml of blood.

[b]Using dye exclusion test (Trypan blue).

The gelatin method is improved by the use of fresh gelatin, a 90-minute separation time, silicon-treated glassware, and defibrinated blood instead of an anticoagulant. The method appeared to give a good yield of WBC, but the RBC counts were consistently high except in Experiment 1.

Isolation Experiments Using Dextran-Hypaque [7]

Experiment 1. Rabbit blood collected in EDTA was layered over gradients in the proportion of 1 part of blood to 1 part of gradient and allowed to stand at room temperature for 90 to 120 minutes to achieve separation. Three gradients were used: (1) 10 parts 33.9% Hp + 20 parts 6% Dextran; (2) 10 parts 33.9% Hp + 25 parts 6% Dextran; and (3) 10 parts 33.9% Hp + 22 parts 9% Dextran. After separation, the leucocyte layer was removed and centrifuged for 10 minutes at 400 x g. The leucocyte pellet was resuspended in HBSS, recentrifuged, and resuspended in MEM-S medium. All three preparations had a very high RBC count (RBC/WBC = 10 to 25).

Experiment 2. Rabbit blood collected in heparin was mixed with 6% Dextran in MEM in the proportion of 3 parts blood to 2 parts Dextran. The mixture was allowed to stand in an ice bath for 60 minutes. The white cell layer was removed and was counted for white and red cells and for viability of the white cells. The results are tabulated below:

Sample	WBC[a]	% Recovery WBC	% Viable	RBC/WBC
1	2.44	57	100	0.89
2	1.51	35	100	0.97

[a]WBC x 10^{-6} recovered/ml whole blood.

Varying quantities of PHA-P were added to aliquots of 3×10^6 WBC in 5ml of MEM containing 5% fetal calf serum, 2 mM glutamine and 250 units/ml each of penicillin and streptomycin contained in Falcon plastic culture flasks. The cultures were incubated at 37°C for 46 hours; 0.5 μc of 2-^{14}C-thymidine was then added to each sample, and the cultures were incubated for an additional 2 hours. The cells were harvested by centrifugation, washed twice with HBSS containing a 100-fold excess of unlabeled thymidine, dissolved in 1 ml of NCS Solubilizer, and counted for radioactivity. The results shown in the table below clearly indicate that the cells were no longer viable at the time the labeled thymidine was added.

Sample	PHA[a]	CPM[b]
1	0	143
2	0.02	115
3	0.02	98
4	0.10	96
5	0.10	106
6	0.20	121
7	0.20	102

[a]One vial of Difco PHA-P was reconstituted in 5 ml of water as directed, and the indicated number of milliliters were added to each culture tube.

[b]Counts per minute were corrected for background.

Experiment 3. Dextran separations were carried out as described previously in three kinds of containers: Falcon plastic disposable tubes, nonsiliconized glass tubes, and siliconized glass tubes. After the separation, all tubes were examined for red cells adhering to the walls of the tubes and interspersed in the upper layer (plasma layer) containing the white cell suspension. All tubes had red cells present in the plasma layer. The red cells adhering to the walls of the tubes frequently remained when the white cell suspension was removed, but when red cells were present within the suspension, the resultant white cell preparation always contained a high red cell count.

Further experiments with Dextran were not done because of the high red cell contamination and because the Ficoll-Hypaque method was recommended by Mansfield [14].

Isolation Experiments Using Methylcellulose Hypaque [7]

The general procedure for this method is as follows. All glassware are sterile and silicon-treated. Rabbit blood collected in EDTA is layered over the gradient (10 parts 33.9% Hp + 16 parts 2% MC) in a ratio of 1 part blood to 1 part gradient. The preparation is incubated at room temperature until the plasma layer separates from the RBC layer. The white cells are harvested from the plasma layer and washed with 4 volumes of HBSS for each volume of plasma. Cells are resuspended in MEM-S medium and incubated at 37°C. Viability counts are made using Eosin Y instead of Trypan blue. A variety of experiments were conducted in an attempt to find optimum conditions for this procedure.

Experiment 1. The general procedure was followed except that the wash step was omitted, resulting in heavy platelet contamination. Viability counts were made immediately after isolation. The results are tabulated below.

Sample	WBC[a]	% Recovery WBC	% Viable	RBC / WBC
1	4.68	24	100	0.66
2	6.10	31	100	0.70
3	6.95	35	100	0.69
4	2.91	22	100	0.55

[a] WBC x 10^{-6} recovered/ml whole blood.

Experiment 2 (effect of varying ratio of MC to Hp). The usual procedure was followed except that the MC:Hp ratio was varied. Viability counts were made after 24 hours in culture. The results were as follows.

Sample	MC:Hp	WBC[a]	% Recovery WBC	% Viable	RBC / WBC
1	12:10	0.82	4.3	47	1.02
2	12:10	0.65	3.4	51	0.82
3	14:10	0.57	2.9	50	1.38
4	14:10	0.33	1.7	38	0.97
5	16:10	0.53	2.8	38	0.38
6	16:10	0.63	3.3	37	0.42
7	18.10	0.63	3.3	28	0.63
8	18:10	0.48	2.5	30	0.87

[a] WBC x 10^{-6} recovered/ml whole blood.

The ratio 16:10 was selected for use in future experiments.

Experiment 3 (effect of varying MC concentration). The usual procedure was followed except that various concentrations of MC were used. The gradient consisted of 10 parts 33.9% Hp + 16 parts MC. Viability counts were made immediately after isolation. The results are tabulated below.

Sample	MC Concentration (%)	WBC[a]	% Recovery	% Viable	RBC/WBC
1	1.25	1.25	9.4	93	2.76
2	1.25	1.38	10.5	89	3.58
3	1.50	0.83	6.25	86	0.89
4	1.50	0.93	7.0	90	0.96
5	1.75	1.49	11.3	93	1.71
6	1.75	1.47	11.1	87	1.53
7	2.0	0.60	4.5	95	1.05
8	2.0	1.06	8.2	87	1.26

[a]WBC x 10^{-6} recovered/ml whole blood.

The WBC yield using 2% MC was typically severalfold higher than shown above. Thus, 2% MC was chosen rather than 1.75% MC. Also, the latter gave higher RBC/WBC ratios than the former.

Experiment 4 (effect of temperature on MC-Hp system). The usual procedure was followed except that two different temperatures were used for separation. Viability counts were made immediately after isolation. The results are tabulated below.

Sample	Temperature	WBC[a]	% Recovery	% Viable	RBC/WBC
1	25°C	2.63	26.3	98	1.01
2	37°C	1.57	15.7	93	0.69
3	37°C	1.41	16.9	86	0.89

[a]WBC x 10^{-6} recovered/ml whole blood.

A temperature of 25°C was found to provide greater WBC recovery.

Experiment 5 (effect of diluting blood on the MC-Hp system). The usual procedure was followed except that a 10:1 dilution of whole blood with 0.9% saline was used. Viability counts were made after 24 hours. The following tabulation presents the results:

Sample	Dilution (Blood: 9% Saline)	WBC[a]	% Recovery	% Viable	RBC / WBC
1	Whole	1.70	18.3	38	0.20
2	Whole	0.72	7.8	53	1.67
3	10:1	1.64	17.7	24	1.98
4	10:1	1.08	11.7	30	4.41

[a] WBC x 10^{-6} recovered/ml whole blood.

Whole blood will be used in future experiments.

Experiment 6 (effect of osmolar concentration of the gradient). Two solutions of Hp were used, 33.9% and 29.75% Hp + 4% NaCl. Both solutions (10 parts) were mixed with MC (16 parts) and used in the standard procedure. Viability counts were made after 24 hours. The results are tabulated as follows.

Sample	Hp Solution	WBC[a]	% Recovery	% Viable	RBC / WBC
1	Hp	0.57	14.8	34	1.92
2	Hp	0.44	11.5	40	1.08
3	Hp	0.41	10.7	37	1.55
4	Hp/salt	0.67	17.4	48	4.20
5	Hp/salt	0.67	17.4	55	1.65
6	Hp/salt	0.51	13.3	43	2.85

[a] WBC x 10^{-6} recovered/ml whole blood.

The MC-Hp system provides good RBC removal and separation of large amounts of blood. The results of these experiments indicate that if a large amount of blood is separated in one tube, the recovery of WBC

increases. However, this requires an increased separation time, which may be a cause of the WBC clumping observed after a 2-hour separation time on the gradient.

Experiment 7 (effect of defibrination). Rabbit blood was defibrinated with wooden sticks [18]. Blood was also collected in EDTA. The usual separation procedure was then followed. The majority of red cells from the defibrinated blood settled out of suspension as expected, but a clearly defined plasma layer was not evident due to extensive hemolysis. No further work was done with this preparation.

White cells recovered from the EDTA blood were washed with MEM (instead of HBSS), resuspended in the usual medium, and cultured for 48 hours. The white cells in the plasma layer from Sample 3 were collected in two parts (the upper half and the lower half) in an effort to demonstrate a red cell gradient in the plasma layer. The data in the table below clearly show that the ratio of red cells to white cells in the lower half of the plasma layer is nearly double that in the upper half. This is partially attributable to the higher white cell count in the upper half. The marked decline in viability is also seen in these data.

Sample	WBC[a]	% Recovery	% Viable			RBC
			0 hr	24 hr	48 hr	WBC
1	0.68	10.1	94	52	13	2.19
2	0.69	10.3	99	57	28	2.13
3(upper)	0.56	8.3	98	55	44	1.42
3(lower)	0.37	5.5	96	61	37	2.77
3(total)	0.93	13.8	--	--	--	1.92

[a]WBC x 10^{-6} recovered/ml whole blood.

Experiment 8 (effect of duration of centrifugation during wash). Rabbit blood was collected in EDTA and separated in the usual way except that the white cells were harvested from the plasma layer by centrifugation at 400 x g for 10 minutes, and washed by resuspending in MEM and centrifuging at 400 x g for either 5 or 10 minutes. The results are tabulated below.

Sample	Time of Centrifugation	WBC[a]	% Recovery	% Viable	RBC / WBC
1	5 minutes	0.68	10	72	2.38
2	5 minutes	0.78	12	73	2.36
3	10 minutes	0.88	13	77	2.11
4	10 minutes	0.55	8	69	2.29

[a]WBC x 10^{-6} recovered/ml whole blood.

These data show that the duration of the wash centrifugation has no effect on the white cell yield or on the red cell contamination.

One possible explanation for the generally low yield of white cells in these and previous experiments, as well as for the rapidly declining viability when various white cell preparations were cultured, is that in comparison to human leucocytes, rabbit leucocytes are inherently more fragile or are made so due to the conditions of isolation and culture This experiment was undertaken to learn the minimum time of centrifugation required to recover the maximum number of white cells during the wash step, based on the hypothesis that minimum exposure to stress would maximize the yield of viable cells. The data obtained from this experiment do not support this hypothesis.

Experiment 9 (effect of sodium and ammonium heparin). Rabbit blood was collected in both ammonium and sodium heparin at 10 U/ml of whole blood. The separations were carried out in the usual way. In addition, a longer separation was included for the blood collected in sodium heparin.

The blood collected in ammonium heparin gave a poor separation due to hemolysis and was discarded. The table below shows the results of the separation of blood collected in sodium heparin.

Sample	Separation Time (min)	WBC[a]	% Recovery	% Viable (24 hr)	RBC / WBC
1	70	0.24	3.3	61	6.83
2	70	0.26	3.6	85	8.27
3	120	1.72	24.0	63	2.72
4	120	1.56	22.0	56	2.03

[a]WBC x 10^{-6} recovered/ml whole blood.

These data show that a longer separation time improves both the white cell yield and the red cell contamination. The higher viability after 24 hours of culture may indicate that heparin is less harmful than EDTA.

Summary of Viability Data from Methylcellulose-Hypaque Experiments. Cell viability is almost always between 90 and 100% immediately after separation (Experiments 1, 3, 4, and 7, but it decreases steadily over a period of 3 days after separation (see following tabulation). The cells appear to be enriched in polymorphonucleocytes, which are known not to survive in culture. The following tabulation summarizes survival data from Experiments 2, 5, 6, 7, and 9. Counts were made at 24, 48, and 72 hours. Only samples separated from whole blood using 2% MC and 33.9% Hp (16:10) are included.

Experiment	Sample	% Viable			RBC/WBC		
		24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
2	5	38			0.38		
	6	37			0.42		
5	1	38	12	9.5	0.20	0.36	0.13
	2	53	14	2.8	1.67	0.17	0.23
6	1	34	21	10	1.92	0.30	0.07
	2	40	26	26	1.08	0.13	1.05
	3	37	7.5	3	1.55	0.23	0.03
7	1	52	13				
	2	57	28				
	3U	55	44				
	3L	61	37				
9	1	61					
	2	85					
	3	63					
	4	56					

This method was abandoned due to the poor longevity of the white cells and because of the research of Mansfield and Wallace [14] who indicated that white cells can be maintained for at least 72 hours by using RPMI-1640 medium supplemented with 5 to 10% fresh, heat-inactivated autologous rabbit plasma.

The Procedure of Mansfield and Wallace

The procedure described below is essentially the method of Mansfield and Wallace, [14] as clarified by personal conversation with Dr. Mansfield by telephone and by a visit to his laboratory at the University of Louisville School of Medicine, Louisville, Kentucky. Other minor modifications have also been made as a result of numerous experiments in our laboratory using their method.

Rabbit blood was drawn by cardiac puncture using a 19-gauge needle attached to a 50-ml syringe containing 1,000 U of heparin or 20 U of heparin/ml of whole blood based on a 50-ml blood volume. However, the amount of blood drawn varied from rabbit to rabbit, with the result that the heparin concentration varied from 20 to 38 U/ml. Aliquots of blood were reserved for analysis of whole blood and for making heat-inactivated plasma as a medium supplement.

Leucocytes were separated from 40 to 60 ml of blood. The blood was centrifuged in siliconized tubes at 160 x g for 15 minutes. The platelet-rich plasma was removed, and the volume was replaced with HBSS.

Aliquots (10 ml) of this blood were then transferred to sterile screw-cap culture tubes (25 x 150 mm) and diluted with 25 ml of HBSS. A 10-ml gradient (1 part 34% Hypaque + 2.4 parts 9% Ficoll) was then layered beneath the diluted blood with a 10-gauge needle. The tubes were then centrifuged at 400 x g for 25 to 30 minutes.

After centrifugation, the clear upper layer consisting of HBSS and platelets was removed by aspiration. The white cell layer formed at the density boundary was transferred to 50-ml screw-cap culture tubes using a sterile disposable pipette, and the cells were resuspended in a fivefold excess of HBSS. The cells were centrifuged at 400 x g for 5 minutes, the supernatant was poured off, and the cells were resuspended by aspiration in RPMI-1640 medium containing per ml 100 U of penicillin, 100 μg of streptomycin, 60 μg of tylosin, and 2 μmoles of glutamine. The cells were then incubated for 2 hours at 37°C.

Viability counts were then done on the isolated cells; 0.1 ml of 1% eosin Y in ethanol was placed in a test tube and then dried. A few drops of cell suspension were added, and the nonstaining viable cells were counted in a hemocytometer. Nonviable cells stained bright pink.

The concentration of viable leucocytes was brought to 1.0×10^6/ml by dilution with medium. Also at this time, heat-inactivated autologous plasma was added to the medium to a concentration of 10%. The plasma supplement was prepared by centrifuging whole blood for 1 hour at 400 x g, removing the plasma layer, and heating it for 30 minutes in a 56°C water bath.

The cell suspensions were then aliquoted to screw-cap culture tubes (16 x 125 mm) in 2-ml volumes (i.e., 2×10^6 viable leucocytes/culture.

To each culture was added 25 μg of PHA in 0.1 ml of HBSS. The cells were gassed with 5% CO_2 in air, capped tightly, and incubated at 37°C for 22 hours.

After 22 hours, the various radioactive substrates were added to 12 replicate cultures in the following order:

Tubes	Labels Added (in 0.1 ml HBSS)
1, 2, 3	2 μCi ^{3}H-thymidine + 1 μCi ^{14}C-uridine
4, 5, 6	1.5 μCi ^{3}H-leucine + 1 μCi ^{14}-choline
7, 8, 9	" "
10, 11, 12	" "

The cultures were then gassed with 5% CO_2 in air, recapped, and incubated for an additional 22 hours.

After 22 hours, samples 1 to 6 were harvested. Samples 7 to 12 were washed twice with HBSS containing a 100-fold excess of unlabeled leucine and choline by centrifuging at 400 x g for 5 minutes for each wash step, and then 2 ml of fresh medium was added. The cells were then gassed and incubated for an additional five hours (Tubes 7 to 9) or 24 hours (Tubes 10 to 12). At these times, the indicated cells were harvested.

Cells were harvested in the following manner. The cultures were diluted with 5 ml of cold 0.15 M NaCl and centrifuged at 400 x g for 5 minutes. The supernatant was decanted, and the cells were washed again in the same manner. The pellet was precipitated with 5 ml of cold 5% TCA and allowed to stand at 4°C for at least 20 minutes. After centrifuging at 400 x g for 10 minutes and decanting the supernatant, the TCA precipitate was dissolved in 1.0 ml of NCS Solubilizer, transferred to scintillation vials in 10 ml of counting fluid (6 g PPO + 75 mg POPOP per liter toluene), and counted in a Searle Analytic Mark III Liquid Scintillation Counter with external standard for dual-label counting. In addition to the teletypewriter printout, the data were also recorded on punched paper tape for computer processing by the Institute's CDC-6400. All counting data are recorded as disintegrations per minute (DPM) as corrected for counting efficiency in the dual-label mode.

Phytohemagglutinin Standardization

Newly purchased Difco PHA-P was used. The contents of six vials were pooled and dissolved in 30 ml of saline. Aliquots of 5 ml were dispensed into serum vials and stored frozen at 20°C until needed.

This PHA-P preparation served for all the remaining experiments in this research.

The PHA-P was first standardized using normal rabbit leucocytes prepared and cultured using the procedure just described except that varying amounts of PHA-P were added and only ^{3}H-thymidine uptake was measured. The mitogenic effect of PHA-P is evident from the data in the accompanying table and is illustrated in Figure 1.

PHA-P (μg/ml)	^{3}H-Thymidine Uptake[a] (DPM $\pm$ S.D.)
0	8284 $\pm$ 1524
2.5	8534 $\pm$ 1305
5.0	25487 $\pm$ 2958
12.5	108064 $\pm$ 10249
50.0	155256 $\pm$ 95111

[a]Average of three values.

The PHA-P concentration of 12.5 μg/ml of culture medium was selected as the standard amount of PHA-P to be used in all subsequent experiments in this research. These data closely approximate those obtained by Mansfield and Wallace, particularly in regard to the low uptake when PHA-P is absent. When this same experiment was carried out earlier using culture conditions shown by others to be suitable for culturing human leucocytes, we obtained higher thymidine uptake in the absence of PHA-P and less stimulation with PHA-P than was found in the present experiment. Mansfield and Wallace [14] state that this effect is primarily due to the use of RPMI 1640 medium containing heat-inactivated autologous rabbit plasma. Thus, the results of our work are consistent with this interpretation.

Preliminary Lead and Cadmium Experiment

This preliminary experiment was undertaken to test all phases of the separation and culture procedures as detailed previously, to mimic the subsequent experiments on exposure of rabbits to lead and cadmium for one and three weeks, and to determine if three samples could be handled simultaneously. We felt it would also be desirable to gain an impression of the metabolic effects of lead and cadmium prior to initiating the main experiments. The procedure differed in several ways from the methods finally adopted. The lead and cadmium animals were treated daily for two weeks; the total culture period was 48 hours instead of 44 hours; the medium contained 5% heat-inactivated autologous rabbit plasma rather than 10%; during the degradation period the plasma supplement was not included; an extra degradation period of 2-1/2 hours was

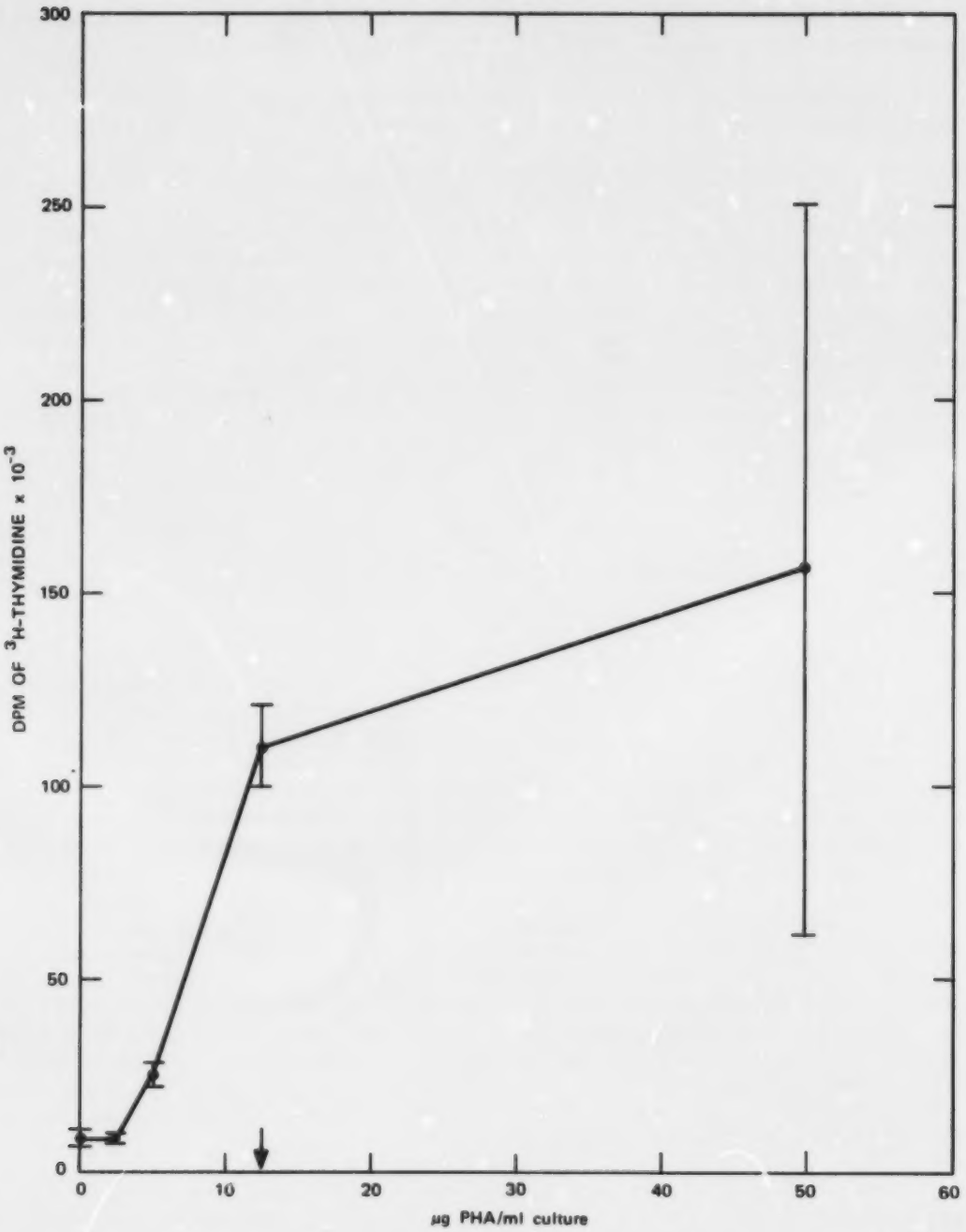

FIGURE 1 PHA STANDARDIZATION

added and the 5-hour period was increased to 5-1/2 hours; the various labels were not combined in ^{14}C-^{3}H pairs but each was added separately to its own set of triplicate culture tubes; and ^{3}H-uridine was used instead of ^{14}C-uridine.

The results demonstrated that the entire procedure was workable and that three blood samples could be handled simultaneously.

As shown in Table 15, the level of incorporation of thymidine was approximately the same as in the PHA standardization experiment.

The incorporation of thymidine into leucocytes from the lead-treated animal was 24% higher than in the control, but because there were only two samples in each group, the difference is not statistically significant. Thus, the effect of lead treatment on DNA synthesis is unclear from this experiment. In contrast, the incorporation of uridine was over twice that in the control, suggesting a stimulation of RNA synthesis. In the white cells from the cadmium-treated animal, the incorporation of both thymidine and uridine was very low, indicating a severe depression of DNA and RNA synthesis. These differences can also be seen by calculating the ratio of uridine to thymidine incorporation in the three animals:

	Control	Lead	Cadmium
Uridine/thymidine	0.360	0.593	1.254
Choline/leucine	0.835	1.122	17.87

Protein biosynthesis in the lead-treated animal is very similar to that in the control animal, as judged by leucine incorporation. The incorporation of leucine in the cadmium-treated rabbit, however, is significantly lower than in the control and is consistent with the diminished biosynthesis of DNA and RNA. The biosynthesis of phospholipid in both treated animals is significantly higher than in the control. These differences are reflected in the ratio of choline to leucine incorporation in the three animals, as shown above.

The degradation of protein and phospholipid is illustrated in Figures 2 and 3, in which the data are normalized to 100% at zero time. Here it can be seen that although the average retention of leucine in the lead-treated animal was higher than in the control, the differences are not statistically significant, indicating that lead treatment has a negligible effect on the turnover of protein in leucocytes. On the other hand, cadmium treatment markedly accelerated the rate of protein turnover during the first five hours of post-incubation, but had no effect during the remainder of the incubation period.

Phospholipid degradation in both the control and lead-treated animals was negligible during the first 5-1/2 hours. Thereafter, the rate

Table 15

INCORPORATION OF LABEL IN
PRELIMINARY LEAD AND CADMIUM EXPERIMENT

Label		Control	Lead	Cadmium
^{3}H-Thymidine		97041[a] ± 15048	148953 ± 21649	1366 ± 188
^{3}H-Uridine		34948 ± 2598	88383 ± 10956	1713 ± 771
^{3}H-Leucine	0 hr	22210 ± 1890	24573 ± 2345	1883 ± 383
	2.5 hr	19247 ± 1360	21951 ± 4209	1037 ± 211
	5.5 hr	16718 ± 4738	19678 ± 3271	775 ± 235
	24 hr	10895 ± 1890	13765 ± 7?1	786 ± 170
^{14}C-Choline	0 hr	18535 ± 1335	27573 ± 2095	33649 ± 1777
	2.5 hr	17521 ± 848	28211 ± 3314	28551 ± 5341
	5.5 hr	17665 ± 748	29783 ± 2765	21002 ± 1281
	24 hr	14092 ± 4318	21802 ± 1638	12750 ± 1595

[a]Entries are DPM ± Standard Deviation.

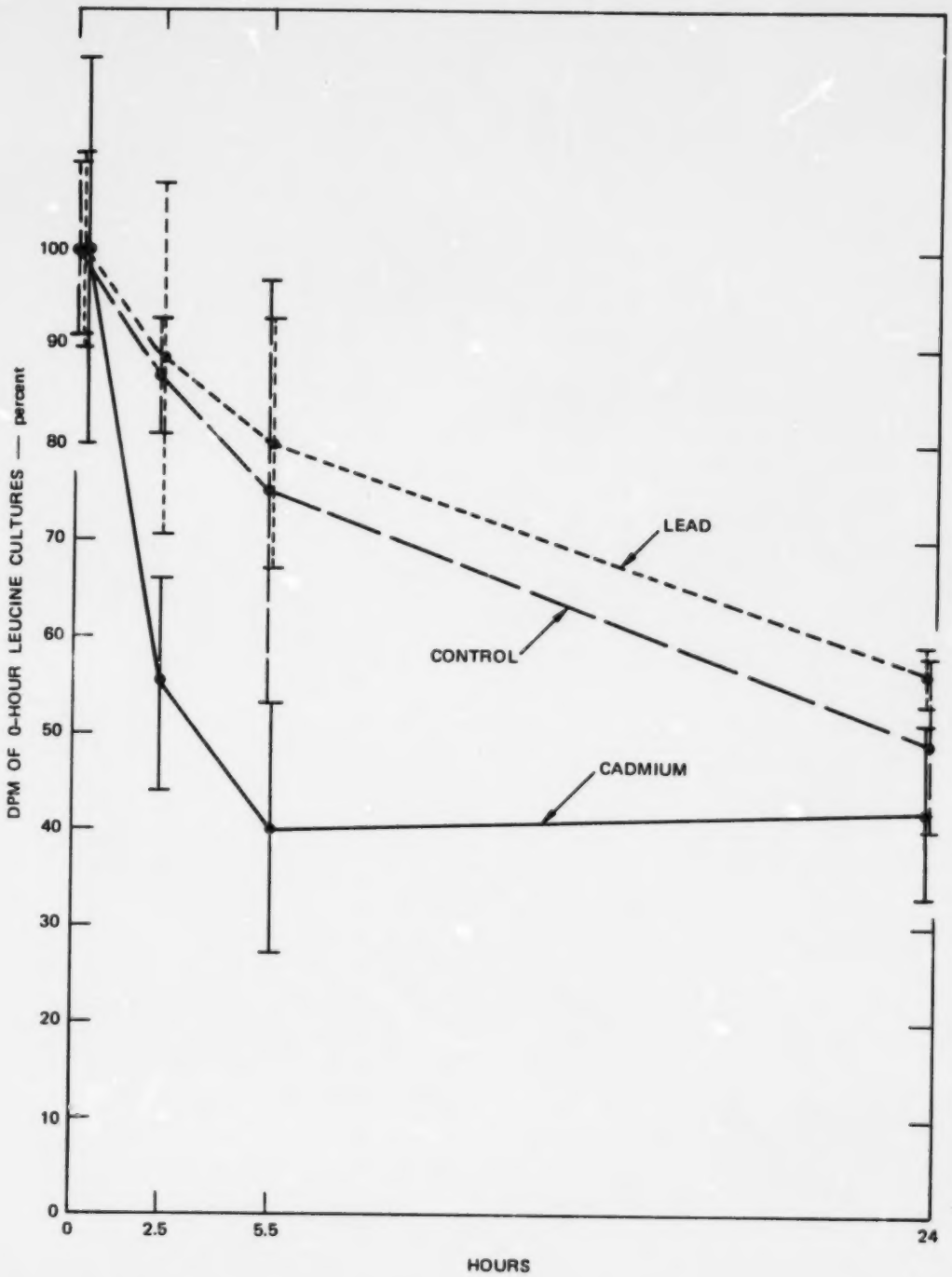

FIGURE 2 LEUCINE DEPLETION IN PRELIMINARY LEAD AND CADMIUM EXPERIMENT

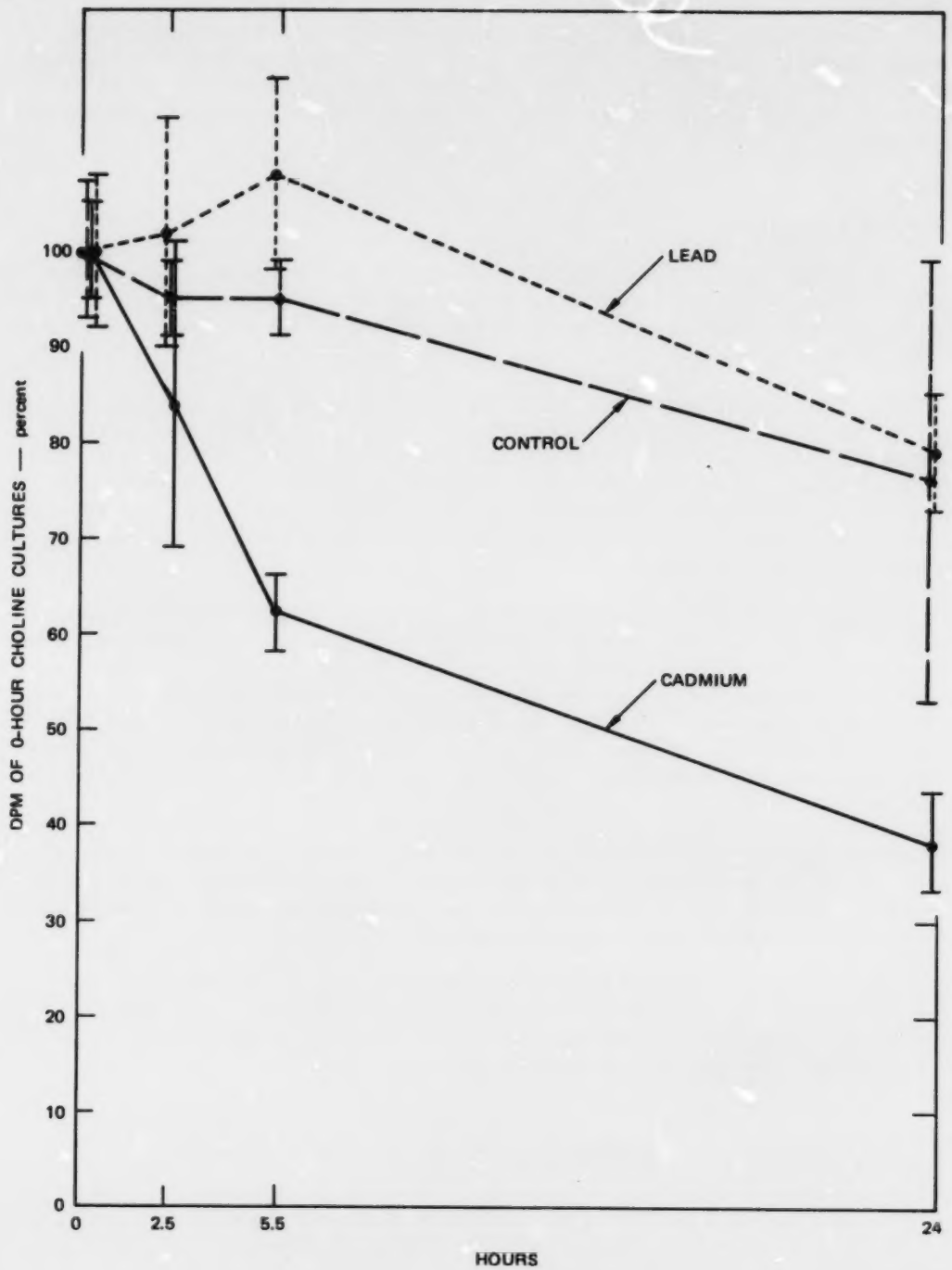

FIGURE 3 CHOLINE DEPLETION IN PRELIMINARY LEAD AND CADMIUM EXPERIMENT

of degradation was very similar in both animals. Thus, the rate of phospholipid turnover in lead-treated animals is probably very similar to that in the control. Throughout the incubation period, the lead-treated animal had significantly more labeled phospholipid than the control.

In contrast, phospholipid degraded rapidly in the cadmium-treated animal during the first 5-1/2 hours and then slowed during the remainder of the incubation period. Thus, cadmium treatment resulted in an increased rate of phospholipid turnover throughout the 24-hour incubation period.

Control Experiment

A second full-scale trial of the entire procedure was undertaken to gain further experience prior to conducting the main experiments and to determine the reproducibility of the experimental data derived from several rabbits.

The results (Tables 16 and 17) show a two- to three-fold variation in the level of incorporation in various samples. These data parallel our earlier impression of the variability of the leucocyte preparations from seemingly identical rabbits. This variability was observed both in the efficacy of the separation of white cells from blood and in the subsequent culturing, even though the procedures were carried out as identically as possible.

Although the incorporation of ^{3}H-thymidine (Table 16) is somewhat higher than in the two previous experiments, the rate is comparable and demonstrates again the mitogenic effect of PHA in stimulating DNA synthesis. Similarly, the uptake of ^{14}C-uridine (Table 16) indicates the concomitant synthesis of RNA. Uridine incorporation in this and the previous experiment cannot be directly compared because different uridine preparations were used. Although 1 μc was added to each tube in both experiments, this amount represents 35.7 μmol in the previous experiment and 17.5 μmol in this one. However, by calculating the uridine/thymidine ratio (shown below), some of the variability among rabbits is removed. This procedure is discussed and documented fully in the next section. The ratios presented here can be compared later with the one-week and three-week exposure experiments.

The levels of incorporation of ^{3}H-leucine and ^{14}C-choline (Table 17) demonstrate the synthesis of protein and phospholipid, respectively. The ratio of choline to leucine in the three animals (shown below) is somewhat higher than in the previous experiment.

Animal	Uridine/Thymidine	Choline/Leucine
1	0.246	1.338
2	0.518	1.113
3	0.627	1.609
Mean	0.464	1.353
SD	$\pm$0.196	$\pm$0.248

Table 16

INCORPORATION OF ^{3}H-THYMIDINE AND ^{14}C-URIDINE IN LEUCOCYTES FROM THREE CONTROL ANIMALS

Animal	^{3}H-Thymidine (DPM)	^{14}C-Uridine (DPM)
1	173778	42776
2	129296	66954
3	98649	61822
Mean	134484	57184
SD	±38457	±11904

Table 17

INCORPORATION OF ^{3}H-LEUCINE AND ^{14}C-CHOLINE IN LEUCOCYTES FROM THREE CONTROL ANIMALS

	^{3}H-Leucine (DPM)			^{14}C-Choline (DPM)		
Animal	0 hr	5 hr	24 hr	0 hr	5 hr	24 hr
1	19001	13301	9026	25442	15745	9444
2	21926	15917	8385	24401	20069	7825
3	61442	50127	23376	98859	71530	24367
Mean	34123	26448	13595	52705	35781	13879
SD	±20804	±17982	±8253	±38266	±27460	±8498

Student t-Test

	DF	t	P
^{3}H-Leucine			
5 hr vs 0 hr	16	0.837	58.5
24 hr vs 0 hr	16	2.752	98.6
^{14}C-Choline			
5 hr vs 0 hr	15	1.057	<90
24 hr vs 0 hr	15	2.974	>99

As shown in Table 18, if the incorporation of label at zero hours is normalized to 100%, then the average degradation rate of protein is nearly the same as that of phospholipid. When the Student's t-test is applied to the individual normalized figures, the differences between the 5-hour and the 0-hour values and between the 24-hour and the 0-hour values become more statistically significant than shown in Table 17. This is particularly true for the 5-hour ^{3}H-leucine data, which in Table 17 is not statistically different from the 0-hour control but which becomes 95% using the normalized values as shown in Table 18. These data are summarized in the table below.

	Raw Data Table 17		Normalized Data Table 18	
^{3}H-Leucine	t	P	t	P
5 hr vs 0 hr	0.837	58.5	6.328	100.0
24 hr vs 0 hr	2.752	98.6	11.667	100.0
^{14}C-Choline				
5 hr vs 0 hr	1.057	<90	5.684	99.9
24 hr vs 0 hr	2.974	>99	19.438	99.9

Although the use of $^{14}C/^{3}H$ ratios and normalization of the degradation data remove much of the variability encountered in this experiment, the one- and three-week experiments were carried out with six rabbits in each group, with the expectation of improving the statistical evaluation of the biosynthetic data.

One-Week and Three-Week Lead and Cadmium Experiments

Animal Data

The animals used in the one-week and three-week exposure experiments were adult New Zealand white rabbits of mixed sex (Tables 19 and 20), weighing 2.66 to 5.39 kg. They were given daily intraperitioneal injections of saline (control animals) or aqueous solutions of lead acetate or cadmium chloride. The metal dosages were determined from intraperitoneal toxicity (LD_{50}) studies and from repeated i.p. injection studies conducted earlier. The dosages were established as 10 mg/kg for lead acetate and 1 mg/kg for cadmium chloride.

	Lead	Cadmium	Units
Salt	10.0	1.0	mg/kg
Metal	5.46	0.492	mg/kg
Metal	26.4	4.38	μmol/kg

Table 18

DEGRADATION RATE OF PROTEIN AND PHOSPHOLIPID
IN THREE CONTROL ANIMALS

Animal	^{3}H-Leucine			^{14}C-Choline		
	0 hr	5 hr	24 hr	0 hr	5 hr	24 hr
1	100.0	70.0	47.5	100.0	61.9	37.1
2	100.0	72.6	38.2	100.0	82.2	32.1
3	100.0	81.6	38.0	100.0	72.4	24.6
Mean	100.0	74.7	41.3	100.0	72.2	31.3
SD	± 6.9	±9.8	±13.4	±3.5	±13.5	±9.4

Student t-Test

	DF	t	P
^{3}H-Leucine			
5 hr vs 0 hr	16	6.328	>99.9
24 hr vs 0 hr	16	11.667	>99.9
^{14}C-Choline			
5 hr vs 0 hr	15	5.684	>99.9
24 hr vs 0 hr	15	19.438	>99.9

The lead was administered as a 50% (w/v) aqueous solution. The cadmium was administered at varying concentrations in a constant volume of 1 ml/kg.

During the first three days of treatment, all rabbits appeared to be mildly depressed as indicated by a lack of alertness. At the same time all animals were restless in that they could not seem to find a comfortable position as though they were internally irritated by the injections. The depression continued throughout the 7- and 21-day treatment periods for the lead- and cadmium-treated animals. One animal died after fifteen days of cadmium treatment. The others appeared to be in good health throughout the treatment period.

All animals were weighed initially and at weekly intervals (Tables 19 and 20). All animals, including controls, showed a mild but statistically significant weight loss after one week of treatment.

Table 19

RABBIT BODY WEIGHTS DURING THE ONE-WEEK EXPOSURE EXPERIMENT

Treatment	Sex	Dose (mg/kg)	Body Wt (kg) 0	Body Wt (kg) 1 wk	Percentage Wt. Loss
Control					
1	♀	0	3.87	3.72	3.88
2	♂		3.14	2.95	6.05
3	♀		3.22	3.05	5.28
4	♀		3.77	3.70	1.86
5	♀		3.52	3.52	0.00
6	♀		4.41	4.00	9.30
Avg.			3.66	3.49	4.40
SD			±0.47	±0.41	±3.27
Cadmium					
1	♂	1.0	3.10	2.82	9.03
2	♀		3.76	3.36	10.64
3	♀		4.01	3.72	7.23
4	♀		3.50	3.35	4.29
5	♀		4.34	4.00	7.83
6	♀		3.73	3.31	11.26
Avg.			3.74	3.43	8.38
SD			±0.42	±0.40	±2.54
v					10
t					2.357
P					>95%
Lead					
1	♀	10.0	3.43	2.78	18.95
2	♀		2.94	2.60	11.56
3	♀		3.00	2.54	15.33
4	♀		3.77	3.42	9.28
5	♂		2.66	2.30	13.53
6	♂		3.60	3.12	13.33
Avg.			3.23	2.79	13.66
SD			±0.43	±0.41	±3.30
v					10
t					4.883
P					>99.9%

Table 20

RABBIT BODY WEIGHTS DURING THE THREE-WEEK EXPOSURE EXPERIMENT

Treatment	Sex	Dose (mg/kg)	Body Wt (kg) 0	1 wk	2 wk	3 wk	Percentage Wt. Loss 1 wk	3 wk
Control								
1	♂	0	3.00	2.82	3.03	2.98	6.00	0.67
2	♀		4.59	4.50	4.22	4.35	1.96	5.23
3	♀		4.36	4.27	4.73	4.40	2.06	-0.92
4	♀		4.71	4.80	4.66	4.60	1.91	2.34
5	♀		4.58	4.19	4.21	4.36	8.52	4.80
6	♀		4.07	4.12	4.08	4.05	-1.23	0.49
Avg.			4.22	4.12	4.16	4.12	2.57	2.10
SD			±0.64	±0.68	±0.61	±0.59	±4.06	±2.49
Cadmium								
1	♀	1.0	2.93	2.73	2.56	2.50	6.83	14.68
2	♀		4.52	4.04	4.19	4.10	10.62	9.29
3	♂		3.16	2.90	3.02	3.05	8.23	3.48
4	♀		4.19	4.11	4.18	4.20	1.91	-0.24
5	♀		3.58	3.34	3.24	3.20	6.70	10.61
6	♀		5.39	5.32	4.87	Died	1.30	--
Avg.			3.96	3.74	3.68	3.41	5.93	7.56
SD			±0.92	±0.96	±0.87	±0.72	±3.64	±5.93
v							10	9
t							1.512	2.067
P							<90%	<95%
Lead								
1	♀	10.0	3.46	3.36	3.31	3.26	2.89	5.78
2	♀		4.42	4.30	4.24	4.18	2.71	5.43
3	♀		3.51	3.06	2.86	3.00	12.82	14.53
4	♀		3.57	3.44	3.38	3.29	3.64	7.84
5	♂		3.08	2.68	2.80	2.78	12.99	9.74
6	♂		3.98	3.84	3.77	3.73	3.52	6.28
Avg.			3.67	3.45	3.39	3.37	6.43	8.27
SD			±0.47	±0.57	±0.55	±0.51	±5.03	±3.46
v							10	10
t							1.464	3.546
P							<90%	99.5%

In the one-week experiment, both groups of metal-treated animals showed a statistically significant weight loss compared to the control group, with the lead-treated animals showing the greater loss (Table 19). In the three-week experiment, the lead-treated animals again showed a greater weight loss after one week than did the cadmium-treated animals, but the differences from the control group were not significant (Table 20). However, at the end of one week the animals in the three-week experiment are equivalent to the animals in the one-week experiment so that the weight loss data can be combined for statistical evaluation. The table below shows that at one week, both metal-treated groups had a significant weight loss compared to the control:

	Control	Lead	Cadmium
Average	3.48%	10.05%	7.16%
SD	±3.64	± 5.54	±3.25
v		22	22
t		3.429	2.607
P		>99.5%	>97.5%

In the three-week experiment, the average weight loss was slightly greater at three weeks than at one week, but the difference was not statistically significant. After three weeks, the weight loss of the lead-treated animals, but not of the cadmium-treated animals, was significantly greater than that in the controls.

Blood Data

A sample of whole blood from each rabbit used in the one- and three-week exposure experiments was analyzed for red and white cell counts and hemoglobin and hematocrit determinations. These data, as well as the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) are presented in Tables 21 and 22.

In the one-week experiment, none of the red cell parameters shown in Table 21 differed significantly from control values. Moreover, all of the average values were within the normal limits (tabulated below) for rabbit blood [19].

WBC (cells x 10^{-6}/ml)	4-13
RBC (cells x 10^{-6}/ml)	4500-7000
Hemoglobin (g%)	10.4-15.6
Hematocrit (%)	33-44
MCV (μm^3)	60-68
MCH (g x 10^{12})	19.4-22.6
MCHC (%)	31.3-34.7

Table 21

WHOLE BLOOD DATA - ONE-WEEK EXPERIMENT

Rabbit	WBC[a]	RBC[a]	Hb (g%)	Hematocrit (%)	MCV (μm^3)	MCH (g x 10^{12})	MCHC (%)
			Control				
1	5.0	5450	13.91	41.0	75.2	25.5	29.5
2	9.0	7350	16.79	49.5	67.3	22.8	29.5
3	5.0	6050	9.63	30.0	49.6	15.9	31.2
4	4.7	6280	10.27	31.5	50.2	16.4	30.6
5	8.1	4800	10.32	35.0	72.9	21.5	33.9
6	4.7	5500	12.05	41.5	75.5	21.9	34.5
Avg.	6.1	5905	12.16	38.1	65.1	20.7	31.5
SD	± 1.9	± 877	± 2.75	± 7.3	±12.1	± 3.8	± 2.2
			Lead				
1	11.1	6750	12.23	39.0	57.8	18.1	31.9
2	7.4	5260	12.19	35.0	66.5	23.2	28.7
3	6.3	4250	8.23	27.0	63.5	19.4	32.7
4	4.8	5000	10.02	34.5	69.0	20.0	34.5
5	9.6	4580	10.58	36.5	79.7	23.1	34.5
6	10.6	5700	10.44	34.5	60.5	18.3	33.1
Avg.	8.3	5257	10.62	34.4	66.2	20.4	32.6
SD	± 2.5	± 890	± 1.49	± 4.0	± 7.8	± 2.3	± 2.2
v	10	10	10	10	10	10	10
t	1.706	1.271	1.217	1.074	0.150	0.145	0.823
P	<90%	<80%	<80%	<80%	<50%	<50%	<60%
			Cadmium				
1	13.4	7080	16.23	51.0	72.0	22.9	31.4
2	10.1	4160	-	-	-	-	-
3	10.4	5250	10.45	33.0	62.9	19.9	31.6
4	8.5	5700	10.02	34.5	60.5	17.6	34.4
5	9.4	5550	10.87	37.5	67.6	19.6	34.5
6	16.9	6600	11.62	40.0	60.6	17.6	34.4
Avg.	11.4	5723	11.83	39.2	64.7	19.5	33.3
SD	± 3.1	±1030	± 2.53	± 7.1	± 5.0	± 2.2	± 1.6
v	10	10	9	9	9	9	9
t	3.561	0.329	0.201	0.255	0.051	0.477	1.244
P	99.5%	<50%	<50%	<50%	<50%	<50%	<80%

[a]Cells x 10^{-6}/ml of whole blood.

Table 22

WHOLE BLOOD DATA - THREE-WEEK EXPERIMENT

Rabbit	WBC[a]	RBC[a]	Hb (g%)	Hematocrit (%)	MCV (μm^3)	MCH (g x 10^{12})	MCHC (%)
				Control			
1	3.5	6900	13.09	42.0	60.9	19.0	32.1
2	6.0	5550	12.66	39.0	70.3	22.8	30.8
3	7.0	5900	10.64	34.0	57.6	18.0	32.0
4	8.2	7900	10.92	37.0	46.8	13.8	33.9
5	14.6	5600	9.67	41.0	73.2	17.3	42.3
6	5.7	4950	9.46	36.0	72.7	19.1	38.1
Avg.	7.5	6133	11.07	38.2	63.6	18.3	34.9
SD	± 3.8	±1076	± 1.51	± 3.1	±10.4	± 2.9	± 4.5
				Lead			
1	4.8	4950	10.69	33.5	67.7	21.6	31.3
2	4.95	5250	11.42	34.5	65.7	21.8	30.1
3	5.8	4140	7.61	28.0	67.6	18.4	36.7
4	9.3	4400	10.58	34.5	78.4	24.0	32.7
5	3.4	3000	4.85	20.0	66.7	16.2	41.2
6	3.6	5050	8.22	33.5	66.3	16.3	40.7
Avg.	5.3	4465	8.9	30.7	68.7	19.7	35.4
SD	± 2.2	± 831	± 2.5	± 5.8	± 4.8	± 3.2	± 4.8
v	10	10	10	10	10	10	10
t	1.232	3.006	1.844	2.816	0.854	0.820	0.227
P	<80%	>97.5%	<95%	>97.5%	<60%	<60%	<50%
				Cadmium			
1	5.6	4300	6.25	20.0	46.5	14.5	32.1
2	4.35	6100	9.41	31.0	50.8	15.4	33.0
3	4.7	6050	9.17	31.0	51.2	15.2	33.7
4	5.3	4650	8.60	28.0	60.2	18.5	32.5
5	6.4	5650	8.76	34.0	60.2	15.5	38.8
Died	-	-	-	-	-	-	-
Avg.	5.3	5350	8.44	28.8	53.8	15.8	34.0
SD	± 0.8	± 827	± 1.27	± 5.36	± 6.1	± 1.5	± 2.7
v	9	9	9	9	9	9	9
t	1.277	1.329	3.105	3.654	1.471	1.347	0.298
P	<80%	<80%	>97.5%	>99%	<90%	<80%	<50%

[a]Cells x 10^{-6}/ml of whole blood.

After three weeks of lead treatment (Table 22), the red cell count, hematocrit, and hemoglobin content were all low, and the red cell size had increased, as shown by the elevated MCV value. These data are consistent with a mild hemolytic anemia characteristic of chronic lead poisoning. In the cadmium-treated animals, on the other hand, the hemoglobin content and the hematocrit were depressed even thouugh the red cell count was not significantly lower than that of the controls. This implies that the red cells were smaller in size and had less hemoglobin than the larger control red cells. The hemoglobin concentration, however, was normal as shown by the MCHC value (Table 22).

In the one-week experiment (Table 21), the white cell count from the cadmium-treated animals was significantly elevated compared with the controls. The white cell count from the lead-treated animals was higher than that of the controls, but the difference was not significant and was within the normal range. At the end of three weeks (Table 22), the white cell count from the lead-and cadmium-treated animals did not differ from that of the controls.

Differential counts were done on the white cells from each rabbit. Whole blood smears were prepared and stained by standard methodology and submitted to Veterinary Reference Laboratory, San Jose, California for analysis. The normal white cell distribution is as follows [19]:

Neutrophils (%)	43 (30-50)
Lymphocytes (%)	42 (30-50)
Monocytes (%)	9 (2-16)
Eosinophils (%)	2 (0.5-5)
Basophils (%)	4 (2-8)

In the one-week experiment (Table 23), the distribution in the lead-treated animals was not significantly different from that of controls. However, the cadmium-treated animals had a significantly elevated neutrophil count and a correspondingly low lymphocyte count. None of the average values were significantly outside normal ranges. The lead-treated animals were characterized by several cellular abnormalities, the most predominant of which were polychromasia and anisocytosis. These characteristics were also observed in the control group but to a lesser extent. Polychromasia was seen in only two of the cadmium-treated animals.

At the end of three weeks (Table 24), the differential counts in both metal-treated groups were not significantly different from that in controls, although the average lymphocyte counts are higher and the average monocyte counts are lower than normal values in both treated groups. The average neutrophil count for the cadmium-treated group is low because of one extraordinarily low value. The frequency of cellular abnormalities had diminished markedly. However, anisocytosis was observed in three of the five lead-treated animals. None of the control rabbits exhibited aberrant white cell forms.

DIFFERENTIAL COUNTS FOR ONE-WEEK EXPERIMENT

Rabbit	Neut. (%)	Lymph. (%)	Mono. (%)	Eos. (%)	Baso. (%)	NRBC[a]	Comments
			Control				
1	26	63	10	-	1	-	-
2	39	54	7	-	-	2	b
3	36	57	6	1	1	-	-
4	40	58	2	-	-	-	b,c
5	34	63	3	-	-	-	-
6	46	51	3	-	-	3	b,c
Avg.	37	58	5				
SD	± 6.7	± 4.8	± 3				
			Lead				
1	25	67	5	-	3	-	b,c,d,e
2	36	60	7	-	-	-	b,c
3	17	80	6	-	-	5	b,d
4	49	47	3	1	-	-	b,c
5	45	52	3	-	-	-	-
6	46	50	4	-	-	2	b,c
Avg.	36	59	4				
SD	±13.8	±12.5	± 1.2				
v	10	10	10				
t	0.150	0.305	0.748				
P	<50%	<50%	<60%				
			Cadmium				
1	42	56	2	-	-	-	b,f
2	54	35	11	-	-	-	b(rare)
3	39	50	11	-	-	-	-
4	64	26	9	1	-	-	-
5	51	47	2	1	-	-	-
6	54	45	1	-	-	-	-
Avg.	51	43	6				
SD	± 9.1	±10.9	± 5				
v	10	10	10				
t	3.004	2.996	0.364				
P	>97.5%	>97.5%	<50%				

[a]Nucleated red blood cells/100 WBC, [b]Polychromasia, [c]Anisocytosis, [d]Basophilic stippling, [e]Several macrocytes, [f]Few Howell-Jolley bodies.

Table 24

DIFFERENTIAL COUNTS FOR THREE-WEEK EXPERIMENT

Rabbit	Neut. (%)	Lymph. (%)	Mono. (%)	Eos. (%)	Baso. (%)	NRBC[a]	Comments
			Control				
1	-	-	-	-	-	-	d
2	33	63	4	-	-	-	-
3	40	58	2	-	-	-	-
4	70	20	5	5	-	-	-
5	13	85	2	-	-	-	-
6	55	44	6	-	-	-	-
Avg.	42	54	3				
SD	±21.7	±24	± 1.6				
			Lead				
1	61	38	1	-	-	7	b,c
2	36	60	4	-	-	1	-
3	31	68	1	-	-	-	c
4	29	71	-	-	-	-	-
5	Too few to count		-	-	-	-	c
6	38	62	-	-	-	-	-
Avg.	39	60	1				
SD	±12.8	±13	± 1.6				
v	8	8	8				
t	0.284	0.475	1.547				
P	<50%	<50%	<90%				
			Cadmium				
1	-	-	-	-	-	-	d
2	59	40	1	-	-	-	b,c(severe)
3	55	45	-	-	-	1	-
4	31	67	2	-	-	-	c
5	13	87	-	-	-	1	-
6	-	-	-	-	-	-	d
Avg.	21.6	60	0.75				
SD	±18.6	±22	± 0.96				
v	7	7	7				
t	0.186	0.372	0.961				
P	<50%	<50%	<80%				

[a]Nucleated red blood cells/100 WBC, [b]Polychromasia, [c]Anisocytosis, [d]Not done.

After separation of the leucocytes by the method detailed previously, the white cell yield and the proportion of red and white cells were measured and the viability of the white cells was determined. These data are presented in Tables 25 and 26 for the one- and three-week experiments, respectively.

The predominant features of these data are the generally very high proportion of viable white blood cells obtained and the low yield of white cells. The poor yields contrast with the experience of Mansfield and Wallace [14]. In spite of intense effort and consultation with Drs. Mansfield and Wallace, who generously donated their time, better yields could not be obtained and the cause of the low yields has not been discovered.

Occasionally a very high proportion of red cells are obtained in the white cell preparations. The data show that there is a strong inverse correlation between white cell yield and red cell contamination. Our present view is that blood platelets play an important role in both parameters.

Biosynthetic Data

These experiments were carried out exactly as described previously with the exception that an extra step was included in an attempt to reduce the platelet population during the preparation of the leucocyte suspension. Whole blood was centrifuged at 160 x g for 15 minutes. The platelet-rich plasma was removed by aspiration and an equal volume of HBSS was added. The blood was then worked up as described previously. This procedure typically resulted in the removal of about 20% of the whole blood volume although there was considerable variation, ranging from 10% to 50%. The one-week data are presented in Tables 30 to 35 and the three-week data are in Tables 36 to 41. One feature common to all the data is the variability in the amount of the incorporated label among the different animals. This is consistent with the previous experiment and reinforces the conclusion that it is due to normal variations in the metabolic activities of leucocytes from the individual rabbits. Further support for this conclusion can be drawn from Table 27, in which each animal is ranked according to its level of incorporation of the four radioactive substrates into its leucocytes. It is seen that in most groupings the white cells from one particular rabbit are most active in taking up all four labels, the cells from another animal are least active with respect to the four labels, and cells from the other animals are ranked in between.

A quantitative measure of this correlation is provided by Kendall's coefficient of concordance [20], which indicates that in five of the six groupings this correlation is highly significant.

Table 25

LEUCOCYTE VIABILITY FOR ONE-WEEK EXPERIMENT

Rabbit	WBC[a]	Viable (%)	RBC / WBC	WBC Yield (%)
		Control		
1	0.55	98	3.2	11
2	1.13	98	2.2	12.5
3	0.55	95	4.3	11
4	0.09	97	18.5[b]	1.9
5	0.83	88	1.9	10
6[c]	1.50	99	1.2	32
		Lead		
1	0.67	95	8.2	6.0
2	0.48	98	4.0	6.5
3	1.13	99	5.5	18
4	0.20	95	29.4[b]	4.2
5	0.55	97	5.4	5.7
6	1.73	99	1.2	16.3
		Cadmium		
1	0.53	96	3.5	4.0
2	0.76	96	2.6	7.5
3	2.00	95	0.64	19
4	1.00	95	0.84	12
5	0.55	94	6.7	5.9
6	0.90	96	2.6	5.3

[a]Cells x 10^{-6} isolated/ml whole blood.

[b]Because of the high red cell contamination, the leucocyte preparation was not used.

[c]Leucocytes from this rabbit were used for the first *in vitro* platinum exposure experiment (see next section).

LEUCOCYTE VIABILITY FOR THREE-WEEK EXPERIMENT

Rabbit	WBC[a]	Viable (%)	RBC / WBC	WBC Yield (%)
		Control		
1	0.65	95	0.48	18.6
2	0.41	98	1.1	6.7
3	0.83	97	0.67	11.9
4	0.06	-	24.1[b]	0.7
5	1.53	98	0.30	10.5
6	0.65	98	2.24	11.4
		Lead		
1	0.52	88	4.3	10.8
2	1.12	97	3.1	22.6
3	0.33	95	40.0[b]	5.7
4	0.53	100	3.6	5.7
5	-	-	high[b]	-
6	0.40	100	37.5	11.1
		Cadmium		
1	0.37	87	1.7	6.6
2	0.65	95	1.34	14.9
3	0.50	90	2.0	10.6
4	0.41	100	3.94	7.7
5	0.50	95	1.56	7.8
6[c]	-	-	-	-

[a]Cells x 10^{-6} isolated/ml whole blood.

[b]Because of the high red cell contamination, the leucocyte preparation was not used.

[c]Animal died on 15th day of treatment.

Table 27

A RANKING OF THE RELATIVE ABILITY OF INDIVIDUAL RABBITS TO INCORPORATE FOUR RADIOACTIVE SUBSTRATES[a]

Animal No.	One Week				Three Weeks			
	T	U	L	C	T	U	L	C
				Control				
1	4	4	4	4	1	2	2	3
2	1	1	1	2	4	5	5	4
3	2	2	2	1	5	4	4	5
4	-	-	-	-	-	-	-	-
5	5	5	3	3	3	1	1	1
6	3	3	5	5	2	3	3	2
s	122				128			
W	0.76				0.80			
P	>99%				>99%			
				Lead				
1	2	1	2	4	2	1	1	1
2	3	3	4	5	1	2	3	4
3	4	4	5	3	-	-	-	-
4	-	-	-	-	3	3	2	3
5	5	5	3	1	-	-	-	-
6	1	2	1	2	4	4	4	2
s	74				50			
W	0.46				0.625			
P	<95%				>95%			
				Cadmium				
1	3	3	1	4	1	1	1	1
2	4	5	4	1	3	2	2	2
3	5	4	5	5	4	4	4	3
4	1	2	2	2	2	3	3	5
5	6	6	6	6	5	5	5	4
6	2	1	3	3	-	-	-	-
s	208				132			
W	0.74				0.82			
P	>99%				>99%			

[a]Radioactive substrates thymidine, uridine, leucine and choline are symbolized by T, U, L, and C, respectively. Entries are the ranking, e.g., in the 1-week experiment control animal #1 ranks 4th for all substrates.

Thus, it is valid to normalize the one- and three-week incorporation data by forming the $^{14}C/^{3}H$ ratio for uridine/thymidine and for choline/leucine and comparing the averages for the lead and cadmium groups with the control group. These correlations are presented in Tables 28 and 29.

The most noteworthy feature of Tables 28 and 29 is the elevation in the uridine/thymidine ratio for the one- and three-week cadmium-treated animals. In both experiments, the cadmium ratios are nearly 4.5 times the control ratios. This effect of cadmium treatment is primarily due to the marked diminution in the ^{3}H-thymidine incorporation, as seen in Tables 30 and 36, although ^{14}C-uridine incorporation is also depressed. The decrease in the ratio at three weeks is due primarily to an increased thymidine incorporation relative to the control while the uridine uptake remained nearly the same. Thus, in the one-week cadmium group the thymidine uptake was 6.5 times less than in the controls, but by three weeks the difference was reduced to a factor of 3.3. Cadmium treatment diminishes uridine incorporation by a factor of 1.7 after one week and of 2.5 after three weeks.

The ratios for the one-week lead-treated group exhibit wide variation and reflect the lack of correlation in ranking the rabbits in the incorporating ability, as seen in Table 27. In both the one- and three-week experiments, the uridine/thymidine ratios for the lead-treated animals are not significantly higher than for the controls. At one week, the absolute incorporation of thymidine and uridine in the lead-treated animals are identical to the controls, but at three weeks the incorporation was depressed 41% and 35%, respectively (Tables 30 and 36). The difference between these two figures is not statistically significant, however, and the resultant uridine/thymidine ratio is the same as that of the controls at three weeks.

Leucine and choline incorporation is not affected by either one- or three-week treatment with lead or cadmium. Also, the choline/leucine ratios for both metal-treated animal groups do not differ significantly from that of the controls. These conclusions are apparent from Tables 32, 34, 38, and 40, in which the incorporation data are presented, as well as from Tables 28 and 29, which show the choline/leucine ratios. It is noteworthy that the incorporation of both leucine and choline at three weeks is nearly double the level at one week.

The depletion rate of labeled leucine is unaffected by the lead or cadmium treatments or by the length of treatment. Approximately 55% of labeled leucine remains after 24 hours post-incubation in all cases (Tables 33 and 39).

Choline depletion in the one-week lead- and cadmium-treated animals is somewhat more rapid than in the controls, but the Student's t-test indicates that the differences are not statistically significant (Table 35). The levels of labeled choline remaining after 24 hours in the three-week lead-treated animals and after 5 hours in the three-week cadmium-treated animals are significantly lower than in the controls (Table 41).

CARBON 14 TO TRITIUM RATIOS IN ONE-WEEK EXPERIMENT

	^{14}C-Uridine/^{3}H-Thymidine			^{14}C-Choline/^{3}H-Leucine		
Animal	Control	Lead	Cadmium	Control	Lead	Cadmium
1	0.784	1.206	3.905	2.579	1.217	0.915
2	0.954	0.863	2.200	1.416	1.952	4.207[a]
3	0.511	1.104	4.941	2.603	3.016	2.185
4	-	-	2.528	-	-	1.459
5	0.644	0.483	3.371	2.157	6.005[a]	2.361
6	0.805	0.442	3.010	1.506	1.367	1.937
Avg.	0.740	0.820	3.326	2.052	2.711	2.177
SD	±0.169	±0.349	±0.994	±0.569	±1.972	±1.124
v		8	9		8	9
t		0.461	10.822		0.718	0.225
P		<90%	>99.9%		<90%	<90%

[a]When these values are deleted, the average values remain statistically not significantly different from the average control value.

Table 29

CARBON 14 TO TRITIUM RATIOS IN THREE-WEEK EXPERIMENT

	^{14}C-Uridine/^{3}H-Thymidine			^{14}C-Choline/^{3}H-Leucine		
Animal	Control	Lead	Cadmium	Control	Lead	Cadmium
1	0.290	0.478	(0.275)[a]	1.596	1.390	1.394
2	0.236	0.252	2.360	4.535	1.524	1.892
3	0.370	-	1.397	2.041	-	3.276
4	-	0.455	1.207	-	1.611	1.536
5	0.595	-	1.042	1.300	-	5.638
6	0.218	(4.739)[a]	-	1.832	3.364	-
Avg.	0.342	0.395	1.502	2.261	1.972	2.745
SD	±0.153	±0.124	±0.590	±1.301	±0.932	±1.775
v		6	7		7	8
t		0.504	4.284		0.372	0.492
P		<90%	>99%		<90%	<90%

[a]Values in parentheses not used in computing averages.

Table 30

ONE-WEEK EXPERIMENT--THYMIDINE AND URIDINE INCORPORATION

ANIMAL	H3-THYMIDINE CONTROL	H3-THYMIDINE PB	H3-THYMIDINE CD	C14-URIDINE CONTROL	C14-URIDINE PB	C14-URIDINE CD
1	26050.	43358.	5825.	15724.	52268.	22747.
2	95356.	32041.	4860.	91024.	27655.	10691.
3	74603.	24590.	7958.	38118.	27142.	20471.
4	0.	0.	10021.	0.	0.	25337.
5	11861.	7984.	1384.	7600.	3856.	4666.
6	25277.	115969.	8533.	20357.	51221.	25686.
MEAN	41858.	45699.	6430.	30532.	32770.	18268.
STD DEV	33003.	40436.	3158.	27857.	19358.	8481.

STUDENT T-TEST

		DF*	T	P			DF	T	P
PB	VS CONTROL	26.	.276	21.517	PB	VS CONTROL	26.	.247	19.305
CD	VS CONTROL	30.	4.548	99.992	CD	VS CONTROL	30.	1.773	91.356

*Same as "v" in text on Tables 30 through 41.

Table 31

ONE-WEEK EXPERIMENT--RATIOS OF URIDINE-TO-THYMIDINE INCORPORATION

ANIMAL	C14-URIDINE / H3-THYMIDINE CONTROL	PB	CD
1	.855	1.188	3.707
	.779	1.234	4.144
	.728	1.197	3.913
2	.930	0.000	2.351
	0.000	.843	1.416
	.979	.885	3.216
3	.495	1.224	2.344
	.511	.988	2.710
	.533	1.123	2.702
4	0.000	0.000	2.698
	0.000	0.000	2.354
	0.000	0.000	2.532
5	.692	.485	3.275
	.604	.498	1.892
	.648	.468	5.110
6	1.029	.429	2.520
	.732	.439	3.804
	.700	.461	3.090
MEAN	.730	.819	2.988
STD DEV	.170	.340	.894

STUDENT T-TEST

		DF	T	P			DF	T	P
PB	VS CONTROL	26.	.876	61.106	CD	VS CONTROL	30.	9.288	100.000

Table 32

ONE-WEEK EXPERIMENT--LEUCINE INCORPORATION AND DEPLETION

ANIMAL	H3-LEUCINE CONTROL 0	CONTROL 5	CONTROL 24	PB 0	PB 5	PB 24	CD 0	CD 5	CD 24
1	10804.	10238.	7121.	23877.	21538.	10312.	28367.	24736.	14453.
2	30432.	27632.	26794.	10688.	9453.	7944.	13287.	10218.	8818.
3	18008.	15759.	16324.	9923.	7974.	6087.	11786.	10389.	8461.
4	0.	0.	0.	0.	0.	0.	23212.	15626.	9939.
5	14001.	9700.	7651.	10866.	0.	4878.	6381.	0.	2645.
6	8037.	4383.	2479.	25703.	17679.	13809.	16849.	15431.	9995.
MEAN	15244.	13542.	9571.	16211.	14280.	8606.	16647.	15264.	8952.
STD DEV	7465.	8241.	6956.	7501.	6292.	3389.	7996.	6033.	3811.

STUDENT T-TEST

			DF	T	P
5 HR CONTROL	VS	0 HR CONTROL	27.	.581	43.411
24 HR CONTROL	VS	0 HR CONTROL	25.	2.038	94.778
5 HR PB	VS	0 HR PB	23.	.671	49.100
24 HR PB	VS	0 HR PB	28.	3.579	99.872
5 HR CD	VS	0 HR CD	31.	.551	41.461
24 HR CD	VS	0 HR CD	34.	3.686	99.921
5 HR PB	VS	0 HR CONTROL	27.	.348	26.941
5 HR PB	VS	5 HR CONTROL	23.	.240	18.728
24 HR PB	VS	24 HR CONTROL	26.	.477	36.272
0 HR CD	VS	0 HR CONTROL	30.	.507	38.396
5 HR CD	VS	5 HR CONTROL	28.	.653	48.080
24 HR CD	VS	24 HR CONTROL	29.	.319	24.773

Table 33

ONE-WEEK EXPERIMENT--RATE OF LEUCINE DEPLETION

ANIMAL	H3-LEUCINE CONTROL 0	CONTROL 5	CONTROL 24	PB 0	PB 5	PB 24	CD 0	CD 5	CD 24
1	100.000	94.761	65.912	100.000	90.204	43.187	100.000	87.200	50.951
2	100.000	90.799	68.329	100.000	88.447	74.329	100.000	76.902	66.342
3	100.000	87.514	57.328	100.000	80.351	61.339	100.000	87.471	71.790
4	0.000	0.000	0.000	0.000	0.000	0.000	100.000	67.318	42.820
5	100.000	69.278	54.642	100.000	0.000	44.894	100.000	0.000	32.055
6	100.000	54.538	30.726	100.000	68.782	53.726	100.000	91.580	59.318
MEAN	100.000	79.378	55.089	100.000	82.612	55.495	100.000	82.094	53.883
STD DEV	12.627	16.866	16.919	7.291	9.330	12.726	15.326	15.419	14.402

STUDENT T-TEST

				DF	T	P
5 HR CONTROL	VS	0 HR CONTROL		27.	3.706	99.904
24 HR CONTROL	VS	0 HR CONTROL		25.	7.856	100.000
5 HR PB	VS	0 HR PB		23.	5.226	99.997
24 HR PB	VS	0 HR PB		28.	11.752	100.000
5 HR CD	VS	0 HR CD		31.	3.333	99.776
24 HR CD	VS	0 HR CD		34.	9.303	100.000
0 HR PB	VS	0 HR CONTROL		27.	.000	.000
5 HR PB	VS	5 HR CONTROL		23.	.550	41.263
24 HR PB	VS	24 HR CONTROL		26.	.072	5.711
0 HR CD	VS	0 HR CONTROL		30.	.000	0.000
5 HR CD	VS	5 HR CONTROL		28.	.466	35.120
24 HR CD	VS	24 HR CONTROL		29.	.214	16.790

Table 34

ONE-WEEK EXPERIMENT--CHOLINE INCORPORATION AND DEPLETION

ANIMAL	C14-CHOLINE CONTROL 0	CONTROL 5	CONTROL 24	PB 0	PB 5	PB 24	CD 0	CD 5	CD 24
1	27863.	28630.	18598.	29061.	24854.	9961.	25960.	21239.	12629.
2	43083.	30172.	18212.	20863.	14619.	9441.	55895.	23749.	17626.
3	46881.	43633.	29074.	29924.	18222.	13896.	25745.	17423.	11785.
4	0.	0.	0.	0.	0.	0.	33857.	18021.	8440.
5	30204.	17789.	11542.	65248.	0.	20265.	15062.	0.	3562.
6	12106.	5524.	2597.	35125.	21032.	11560.	32644.	25711.	11402.
MEAN	31238.	25150.	13992.	36044.	20053.	13024.	31527.	21228.	10907.
STD DEV	13029.	13319.	8109.	16024.	4171.	4164.	13937.	4704.	4457.

STUDENT T-TEST

			DF	T	P
5 HR CONTROL	VS	0 HR CONTROL	27.	1.243	77.541
24 HR CONTROL	VS	0 HR CONTROL	25.	4.090	99.961
5 HR PB	VS	0 HR PB	23.	3.067	99.454
24 HR PB	VS	0 HR PB	28.	5.385	99.999
5 HR CD	VS	0 HR CD	31.	2.729	98.963
24 HR CD	VS	0 HR CD	34.	5.979	100.000
0 HR PB	VS	0 HR CONTROL	27.	.882	61.465
5 HR PB	VS	5 HR CONTROL	23.	1.165	74.418
24 HR PB	VS	24 HR CONTROL	26.	.405	31.142
0 HR CD	VS	0 HR CONTROL	30.	.060	4.746
5 HR CD	VS	5 HR CONTROL	28.	1.075	70.855
24 HR CD	VS	24 HR CONTROL	29.	1.360	81.556

Table 35

ONE-WEEK EXPERIMENT--RATE OF CHOLINE DEPLETION

ANIMAL	C14-CHOLINE CONTROL 0	CONTROL 5	CONTROL 24	C14-CHOLINE PB 0	PB 5	PB 24	C14-CHOLINE CD 0	CD 5	CD 24
1	100.000	102.755	66.719	100.000	85.521	34.274	100.000	81.814	48.3[illegible]
2	100.000	70.634	42.272	100.000	70.069	45.252	100.000	42.488	31.833
3	100.000	93.073	62.018	100.000	88.093	46.437	100.000	67.673	45.776
4	0.000	0.000	0.000	0.000	0.000	0.000	100.000	53.226	24.929
5	100.000	58.897	38.212	100.000	0.000	31.058	100.000	0.000	23.646
6	100.000	45.631	21.450	100.000	59.879	32.912	100.000	78.761	34.929
MEAN	100.000	74.078	43.691	100.000	69.914	37.987	100.000	64.792	34.910
STD DEV	12.854	22.068	18.319	7.562	12.080	7.411	16.436	18.766	10.129

STUDENT T-TEST

			DF	T	P
5 HR CONTROL	VS	0 HR CONTROL	27.	3.725	99.909
24 HR CONTROL	VS	0 HR CONTROL	25.	9.382	100.000
5 HR PB	VS	0 HR PB	23.	7.687	100.000
24 HR PB	VS	0 HR PB	26.	22.684	100.000
5 HR CD	VS	0 HR CD	31.	5.743	100.000
24 HR CD	VS	0 HR CD	34.	14.304	100.000
[illegible] HR PB	VS	0 HR CONTROL	27.	.000	.000
5 HR PB	VS	5 HR CONTROL	23.	.526	39.636
24 HR PB	VS	24 HR CONTROL	26.	1.108	72.215
[illegible] HR CD	VS	0 HR CONTROL	30.	0.000	0.000
5 HR CD	VS	5 HR CONTROL	28.	1.215	76.555
24 HR CD	VS	24 HR CONTROL	29.	1.718	90.269

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Table 36

THREE-WEEK EXPERIMENT--THYMIDINE AND URIDINE INCORPORATION

ANIMAL	H3-THYMIDINE CONTROL	H3-THYMIDINE PB	H3-THYMIDINE CD	C14-URIDINE CONTROL	C14-URIDINE PB	C14-URIDINE CD
1	308092.	183953.	278547.	89497.	87840.	76574.
2	125472.	245082.	14675.	29562.	61685.	34630.
3	99858.	0.	5006.	36935.	0.	6994.
4	0.	84936.	19157.	0.	38606.	23121.
5	235633.	0.	2235.	140133.	0.	2329.
6	281430.	2680.	0.	61173.	12781.	0.
MEAN	210094.	124182.	63924.	71460.	46787.	28729.
STD DEV	88063.	104307.	111349.	42221.	29865.	28959.

STUDENT T-TEST

		DF	T	P			DF	T	P
PB	VS CONTROL	24.	2.274	96.781	PB	VS CONTROL	24.	1.685	88.912
CD	VS CONTROL	28.	3.988	99.957	CD	VS CONTROL	28.	3.232	99.686

Table 37

THREE-WEEK EXPERIMENT--RATIOS OF URIDINE-TO-THYMIDINE INCORPORATION

ANIMAL	C14-URIDINE / H3-THYMIDINE CONTROL	PB	CD
1	.310	.500	.339
	.274	.469	.225
	.286	0.000	.265
2	.255	.248	1.841
	.214	.232	1.901
	.235	.283	3.109
3	.382	0.000	1.239
	.402	0.000	1.350
	.324	0.000	1.722
4	0.000	.310	1.434
	0.000	.472	.909
	0.000	.568	1.207
5	.580	0.000	1.305
	.594	0.000	.970
	.611	0.000	.707
6	.229	4.959	0.000
	.223	5.566	0.000
	.195	4.125	0.000
MEAN	.341	1.618	1.236
STD DEV	.144	2.130	.748

STUDENT T-TEST

		DF	T	P			DF	T	P
PB	VS CONTROL	24.	2.318	97.072	CD	VS CONTROL	20.	4.568	99.990

Table 38

THREE-WEEK EXPERIMENT--LEUCINE INCORPORATION AND DEPLETION

	H3-LEUCINE								
	CONTROL			PB			CD		
ANIMAL	0	5	24	0	5	24	0	5	24
1	33351.	31652.	17928.	74468.	0.	39076.	66948.	0.	0.
2	10477.	7549.	5588.	21252.	17478.	12475.	39939.	32263.	18638.
3	21589.	17722.	13648.	0.	0.	0.	14748.	11544.	6681.
4	0.	0.	0.	21818.	16278.	11407.	24317.	0.	13542.
5	46374.	36931.	32217.	0.	0.	0.	7425.	7635.	7211.
6	31779.	24887.	17178.	15997.	10789.	7569.	0.	0.	0.
MEAN	28698.	23748.	16444.	33384.	14520.	18547.	30675.	15258.	12385.
STD DEV	12937.	11573.	8842.	25472.	3351.	14088.	23657.	10828.	5323.

STUDENT T-TEST

			DF	T	P
5 HR CONTROL	VS	0 HR CONTROL	28.	1.184	72.120
24 HR CONTROL	VS	0 HR CONTROL	26.	2.379	99.211
5 HR PB	VS	0 HR PB	18.	2.064	94.629
24 HR PB	VS	0 HR PB	21.	1.708	89.755
5 HR CD	VS	0 HR CD	21.	1.775	90.966
24 HR CD	VS	0 HR CD	23.	2.483	97.783
0 HR PB	VS	0 HR CONTROL	25.	.621	45.996
5 HR PB	VS	5 HR CONTROL	21.	2.185	95.968
24 HR PB	VS	24 HR CONTROL	22.	.447	34.873
0 HR CD	VS	0 HR CONTROL	28.	.290	22.576
5 HR CD	VS	5 HR CONTROL	21.	1.712	89.838
24 HR CD	VS	24 HR CONTROL	21.	1.290	78.888

Table 39

THREE-WEEK EXPERIMENT--RATE OF LEUCINE DEPLETION

	H3-LEUCINE								
	CONTROL			PB			CD		
ANIMAL	0	5	24	0	5	24	0	5	24
1	100.000	94.904	53.757	100.000	0.000	52.474	100.000	0.000	0.000
2	100.000	72.854	53.261	100.000	82.345	56.760	100.000	86.781	46.661
3	100.000	82.393	63.415	0.000	0.000	0.000	100.000	70.279	44.761
4	0.000	0.000	0.000	100.000	74.610	52.285	100.000	0.000	55.691
5	100.000	79.638	69.472	0.000	0.000	0.000	100.000	102.836	97.125
6	100.000	78.313	54.055	100.000	67.442	47.314	0.000	0.000	0.000
MEAN	100.000	81.460	57.615	100.000	71.831	53.182	100.000	88.113	53.846
STD DEV	14.173	15.208	9.663	26.317	9.417	8.410	17.032	23.684	16.188

STUDENT T-TEST

				DF	T	P
5 HR CONTROL	VS	0 HR CONTROL		28.	3.454	99.822
24 HR CONTROL	VS	0 HR CONTROL		26.	9.095	100.000
5 HR PB	VS	0 HR PB		18.	3.386	99.671
24 HR PB	VS	0 HR PB		21.	7.095	100.000
5 HR CD	VS	0 HR CD		21.	1.392	82.186
24 HR CD	VS	0 HR CD		23.	6.771	100.000
5 HR PB	VS	0 HR CONTROL		25.	.000	.000
5 HR PB	VS	5 HR CONTROL		21.	1.286	78.742
24 HR PB	VS	24 HR CONTROL		22.	1.187	75.213
5 HR CD	VS	0 HR CONTROL		28.	.000	0.000
5 HR CD	VS	5 HR CONTROL		21.	.823	58.019
24 HR CD	VS	24 HR CONTROL		21.	.697	50.630

Table 40

THREE-WEEK EXPERIMENT--CHOLINE INCORPORATION AND DEPLETION

	C14-CHOLINE								
	CONTROL			PB			CD		
ANIMAL	0	5	24	0	5	24	0	5	24
1	53231.	42753.	25224.	103540.	0.	38169.	93332.	0.	0.
2	47508.	31199.	25646.	32390.	24531.	14074.	78582.	48614.	30129.
3	43965.	26619.	17702.	0.	0.	0.	48314.	28963.	20793.
4	0.	0.	0.	35151.	21179.	15234.	37339.	0.	19885.
5	60308.	43055.	25551.	0.	0.	0.	41784.	28997.	17880.
6	58269.	39009.	26866.	53869.	24861.	14465.	0.	0.	0.
MEAN	52632.	36527.	24592.	56222.	23398.	21022.	59270.	30731.	22729.
STD DEV	7868.	8560.	3914.	38423.	2965.	12676.	24695.	12113.	8381.

STUDENT T-TEST

				DF	T	P
5 HR CONTROL	VS	0 HR CONTROL		28.	5.384	99.996
24 HR CONTROL	VS	0 HR CONTROL		26.	11.715	100.000
5 HR PB	VS	0 HR PB		18.	3.015	99.256
24 HR PB	VS	0 HR PB		21.	3.589	99.815
5 HR CD	VS	0 HR CD		21.	3.054	99.398
24 HR CD	VS	0 HR CD		23.	4.576	99.987
0 HR PB	VS	0 HR CONTROL		25.	.441	33.717
5 HR PB	VS	5 HR CONTROL		21.	4.168	99.956
24 HR PB	VS	24 HR CONTROL		22.	.966	65.564
0 HR CD	VS	0 HR CONTROL		28.	.993	67.062
5 HR CD	VS	5 HR CONTROL		21.	1.339	80.511
24 HR CD	VS	24 HR CONTROL		21.	.963	65.239

Table 41

THREE-WEEK EXPERIMENT--RATE OF CHOLINE DEPLETION

	C14-CHOLINE								
	CONTROL			PB			CD		
ANIMAL	0	5	24	0	5	24	0	5	24
1	100.000	80.316	47.386	100.000	0.000	36.864	100.000	0.000	0.000
2	100.000	65.672	53.970	100.000	75.737	43.451	100.000	64.320	39.063
3	100.000	60.628	40.319	0.000	0.000	0.000	100.000	59.906	43.636
4	0.000	0.000	0.000	100.000	60.253	43.339	100.000	0.000	50.009
5	100.000	71.391	42.368	0.000	0.000	0.000	100.000	49.293	41.900
6	100.000	67.015	46.154	100.000	46.203	26.771	0.000	0.000	0.000
MEAN	100.000	69.005	46.762	100.000	58.855	38.591	100.000	57.030	44.330
STD DEV	7.884	11.280	6.563	9.540	13.074	9.324	18.826	9.936	5.172

STUDENT T-TEST

			DF	T	P
5 HR CONTROL	VS	0 HR CONTROL	28.	8.722	100.000
24 HR CONTROL	VS	0 HR CONTROL	26.	19.300	100.000
5 HR PB	VS	0 HR PB	10.	8.150	100.000
24 HR PB	VS	0 HR PB	21.	15.586	100.000
5 HR CD	VS	0 HR CD	21.	6.942	100.000
24 HR CD	VS	0 HR CD	23.	10.684	100.000
5 HR PB	VS	0 HR CONTROL	25.	0.000	0.000
5 HR PB	VS	5 HR CONTROL	21.	1.947	93.495
24 HR PB	VS	24 HR CONTROL	22.	2.521	98.054
0 HR CD	VS	0 HR CONTROL	26.	.000	0.000
5 HR CD	VS	5 HR CONTROL	21.	2.521	98.014
24 HR CD	VS	24 HR CONTROL	21.	.969	65.629

One final feature of Tables 28 and 29 is the constancy of the effect of lead and cadmium treatment on the $^{14}C/^{3}H$ ratios compared to the controls. This is illustrated in the table below.

	^{14}C-Uridine/^{3}H-Thymidine		^{14}C-Choline/^{3}H-Leucine	
	1 wk	3 wk	1 wk	3 wk
Control	1.00	1.00	1.00	1.00
Lead	1.11	1.15	0.92	0.87
Cadmium	4.49	4.39	0.86	0.90

Thus, while the relative incorporation of thymidine and uridine in the lead- and cadmium-treated animals does not change compared to the controls in the two experiments, individual incorporation of the two substrates changes markedly from one week to three weeks. Thus, in the control animals, thymidine uptake increases five-fold, compared to a 2.5-fold increase in uridine uptake (Table 30 versus Table 36). In the lead-treated animals, the corresponding values are 2.6 and 1.2, while in the cadmium group the increases are 1.6 and 0.9, respectively (values for the first cadmium-treated animal are not included in the three-week calculations). In each case, the uridine ratio at three weeks is about half that at one week.

In marked contrast, the choline/leucine ratios are virtually constant for all three groups in both the one- and three-week experiments, although the absolute incorporation of labeled choline and leucine at three weeks is approximately double the values at one week (Table 32 versus Table 38 and Table 34 versus Table 40). The incorporation data for the one- and three-week experiments are summarized in Table 42.

In Vitro Platinum Exposure Experiments

Leucocytes from the sixth control rabbit of the one-week exposure experiment were used for the first experiment. Twelve cultures containing 2 x 10^6 viable WBC were prepared in 2 ml of medium. To each was added 1 mg of sodium hexachloroplatinate (IV) ($Na_2PtCl_6 \cdot 6H_2O$) in a 0.1-ml volume of HBSS. The final concentration of the platinum salt was 0.476 mg/ml of culture medium or 0.848 μM. This is equivalent to 165 μg/ml as metallic platinum. The cultures were then gassed with 5% CO_2 in air and incubated for 2 hours at 37°C.

At the end of the 2-hour incubation, each culture was mixed with 2 ml of HBSS and centrifuged at 400 x g for 5 minutes; the supernatant was decanted. The cells from each culture were then washed with 3 ml of HBSS and resuspended in 2 ml of medium and 0.1 ml of PHA in HBSS. At this point, the platinum-treated cells were firmly attached to the wall

Table 42

SUMMARY OF EFFECTS OF LEAD AND CADMIUM TREATMENT[a]

	A One Week		A Three Weeks		B Three Weeks		
	Lead	Cadmium	Lead	Cadmium	Control	Lead	Cadmium
^{3}H-Thymidine	NC	[illegible]↓	41↓	95↓	402↑	172↑	60↑
^{14}C-Uridine	NC	40↓	35↓	77↓	134↑	43↑	57↑
^{14}C-Uridine/^{3}H-Thymidine	NC	309↑	NC	339↑	54↓	52↓	55↓
^{3}H-Leucine	NC	NC	NC	NC	88↑	106↑	84↑
^{14}C-Choline	NC	NC	NC	NC	68↑	56↑	88↑
^{14}C-Choline/^{3}H-Leucine	NC	NC	NC	NC	NC	NC	14↑
^{3}H-Leucine 5 hr	NC	NC	NC	NC	NC	NC	NC
^{3}H-Leucine 24 hr	NC	NC	NC	NC	NC	NC	NC
^{14}C-Choline 5 hr	NC	NC	NC	17↑	NC	16↓	NC
^{14}C-Choline 24 hr	NC	NC	17↑	NC	NC	NC	27↑

[a]Entries show percentage by which the tabulated group differs (↑ = increase, ↓ = decrease) from the control group (A) or from the corresponding one-week group (B), or by which the tabulated 5-hr or 24-hr group differs from the zero-hour group. Differences less than 10% or those not statistically significant are indicated by NC (no change).

of the culture tubes. They did not come off until they were harvested. The untreated control cells, which did not stick as much or as tightly to the culture tubes, were not washed as were the treated cells.

After the addition of PHA, the cultures were incubated, exposed to radioactive substrates, and harvested exactly as described previously, except that no 5-hour pulse was done.

The second *in vitro* platinum experiment was carried out exactly like the first except that an extra set of triplicate cultures were included and treated with 0.238 mg/ml of platinum in addition to treatment at 0.476 mg/ml. The cells for this experiment were from the third control rabbit of the three-week exposure experiment. The platinum-treated cells adhered to the culture tube walls and the controls did not, as in the first experiment.

The third and final *in vitro* platinum experiment was carried out exactly like the first except that each culture contained only 0.5×10^6 viable WBC/ml of medium (one-half of normal). Nine cultures received no platinum salt (controls), nine received 95.2 μg/ml, and nine received 0.476 mg/ml of platinum salt. There was no 5-hour pulse. The cells used in this experiment were from an untreated rabbit. The blood analysis and viability data are presented below.

WBC[a]	2.15
RBC[a]	6450
Hb (g%)	11.79
Hematocrit (%)	43.0
Differential count:	
Neutrophils (%)	14
Lymphocytes (%)	85
Monocytes (%)	1
Eosinophils (%)	0
Basophils (%)	0
Nucleated RBC[b]	1
W C isolated	0.52
Viable WBC (%)	100
RBC/WBC	2.2
WBC yield (%)	24.2

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[a]cells x 10^{-6}/ml of whole blood.

[b]cells/100 WBC.

The results of the first experiment (Tables 43 and 44) show a dramatic depression in the incorporation of thymidine in *in vitro* platinum-treated leucocytes. Thymidine uptake in these cells was only 15% of that

Table 43

INCORPORATION OF LABELED SUBSTRATES INTO IN VITRO PLATINUM-TREATED LEUCOCYTES

First Experiment - Incorporation and Degradation Data

	Tritium		Carbon 14	
Control	Thymidine	Leucine	Uridine	Choline
0 hr	25439 ± 3742	7923 ±2354	20479 ± 2217	12265 ± 3600
5 hr	-	4335 ±1060	-	5690 ± 2072
24 hr	-	2531 ± 635	-	2628 ± 650
Platinum (0.5 mg/ml)				
0 hr	3818 ± 393	5515 ± 684	11153 ± 1004	11331 ± 825
24 hr	-	3269 ± 489	-	4207 ± 1922

Student's t-Test

Substrate	Comparison	v	t	P
Thymidine	Pt vs control	4	9.952	>99.9%
Uridine	Pt vs control	4	6.637	>99%
Leucine	Pt vs control	4	1.702	<90%
Choline	Pt vs control	4	0.438	<90%

Table 44

INCORPORATION OF LABELED SUBSTRATES INTO IN VITRO PLATINUM-TREATED LEUCOCYTES

First Experiment - Percent Incorporation and Degradation

	Incorporation	
Substrate	Percent of Control	Percent Depressed
Thymidine	15.0	85.0
Uridine	54.5	45.5
Leucine	69.6	30.4
Choline	92.4	7.6

	Degradation: Percent of Zero Hour			
	Control		Platinum	
Substrate	5 hr	24 hr	5 hr	24 hr
Leucine	54.7	31.9	-	59.3
Choline	46.4	21.4	-	37.1

of the controls. Uridine uptake was reduced by one-half. These data indicate that platinum treatment markedly interferes with DNA synthesis and also diminishes RNA synthesis.

Choline incorporation, on the other hand, is only slightly affected, with an 8% reduction compared to the controls. Leucine uptake is depressed to 70% of normal, but the difference does not appear to be significant. These data indicate that the in vitro platinum treatment has only a slight effect of phospholipid biosynthesis, while the effect on protein biosynthesis is uncertain. In contrast, the rate at which whole cell protein and phospholipids are catabolized is clearly influenced by the platinum treatment. In the control cells, both constituents show approximately a 50% turnover after 5 hours, whereas in the treated cells a 50% turnover for protein is not achieved until after 24 hours. Similarly, the phospholipid turnover rate is also increased by nearly a factor of two. Thus, while the biosynthesis of protein and phospholipid is virtually unaffected by platinum treatment, the rate of degradation is strongly depressed.

The results of the second experiment (Tables 45 and 46) do not appear to corroborate the results of the first. Both thymidine and uridine uptake are depressed by about 30% in the cells treated with 0.5 mg/ml of platinum, the same dose level as in the first experiment. Similarly, both leucine and choline incorporation are depressed to about the same extent, that is, about 20%. At the lower platinum concentration of 0.25 mg/ml, incorporation of thymidine is lower but the difference is not statistically significant. Thus, the two platinum concentrations are equally effective in inhibiting thymidine and uridine uptake. Degradation studies were not carried out in this experiment.

The results of the third experiment, presented in Tables 47 and 48, demonstrate the inhibition of thymidine and uridine incorporation at both concentrations of platinum treatment. As expected, a greater inhibition is observed with the higher platinum level of 0.5 mg/ml; this is in contrast to the second experiment, in which the two treatment levels did not result in significantly different incorporation. As in the first experiment, platinum inhibits the uptake of thymidine to a greater extent than that of uridine, but the differences are not nearly so pronounced.

A surprising observation is that both platinum treatments appeared to stimulate choline incorporation--by nearly 70% at the lower platinum level and 40% at the higher level. However, these figures are in reference to a single control culture that remained out of three originally prepared. Although it is possible that the values for the control choline tube are abnormally low, it seems unlikely in view of the generally low standard deviations found with the other triplicate cultures (Table 47).

Leucine incorporation is slightly depressed at the 0.1 mg treatment level, but the difference is not significant. At the higher platinum concentration, the incorporation is significantly less by more than 40%.

The degradation rates of protein and phospholipid in this experiment appear to be only slightly retarded compared to the controls. Thus, 60% of both leucine and choline remains in the 0.5 mg platinum-treated cells after 24 hours, whereas about 55% remains in the control.

Choline retention is the same at both platinum concentrations after 24 hours, but at the lower level protein degradation is increased slightly, with 48% of labeled leucine remaining.

Table 45

INCORPORATION OF LABELED SUBSTRATES INTO IN VITRO PLATINUM-TREATED LEUCOCYTES

Second Experiment - Incorporation Data

	Tritium		Carbon 14	
	Thymidine	Leucine	Uridine	Choline
Control	99501 ± 2950	20267 ± 802	36744 ± 4577	43310 ± 2219
Platinum				
0.25 mg/ml	61751 ±19428	- -	24436 ± 4579	- -
0.5 mg/ml	71903 ±11841	15173 ± 1151	24503 ± 2049	35210 ± 2969

Student's t-Test

Substrate	Comparison	v	t	P
Thymidine	0.25 Pt vs control	3	3.604	>95%
	0.5 Pt vs control	4	3.917	>98%
	0.25 Pt vs 0.5 Pt	3	0.751	<50%
Uridine	0.25 Pt vs control	3	2.945	<95%
	0.5 Pt vs control	4	4.237	>98%
	0.25 Pt vs 0.5 Pt	3	0.024	<50%
Leucine	0.5 Pt vs control	4	6.289	>99%
Choline	0.5 Pt vs control	4	3.786	>98%

Table 46

INCORPORATION OF LABELED SUBSTRATES INTO *IN VITRO* PLATINUM-TREATED LEUCOCYTES

Second Experiment - Percent Incorporation

	Incorporation			
	Percent of Control		Percent Depression	
Substrate	0.25	0.50	0.25	0.50
Thymidine	62.0	72.3	38.0	27.7
Uridine	66.5	67.7	33.5	33.3
Choline	-	81.3	-	18.7
Leucine	-	75.0	-	25.0

Table 47

INCORPORATION OF LABELED SUBSTRATES INTO IN VITRO PLATINUM-TREATED LEUCOCYTES

Third Experiment - Incorporation and Degradation Data

	Tritium		Carbon 14	
	Thymidine	Uridine	Leucine	Choline
Control				
0 hours	33055 ± 2718	13367 ± 1118	8813 -	15744 -
24 hours	- -	- -	4874 ±1218	8413 ± 1071
Platinum - 0.1 mg/ml				
0 hours	18200 ± 3924	6352 ± 1005	7739 ± 283	26489 ± 1491
24 hours	- -	- -	3725 409	15410 138
Platinum - 0.5 mg/ml				
0 hours	8531 ± 951	4068 ± 318	5099 ± 370	21757 ± 858
24 hours	- -	- -	3076 ± 65	12818 ± 130

Student's t-Test

Substrate	Comparison	v	t	P
Thymidine	0.1 Pt vs control	4	5.390	>99%
	1.5 Pt vs control	4	14.750	>99.9%
	0.1 Pt vs 0.5 Pt	4	4.148	>98%
Uridine	0.1 Pt vs control	4	7.808	>99%
	0.5 Pt vs control	4	13.093	>99.9%
	0.1 Pt vs 0.5 Pt	4	3.754	>98%
Leucine	0.1 Pt vs control	1	5.370	<90%
	0.5 Pt vs control	2	13.486	>99%
	0.1 Pt vs 0.5 Pt	3	8.431	>99%
Choline	0.1 Pt vs control	1	10.194	<95%
	0.5 Pt vs control	2	9.407	>98%
	0.1 Pt vs 0.5 Pt	3	4.672	>98%

Table 48

INCORPORATION OF LABELED SUBSTRATES INTO IN VITRO PLATINUM-TREATED LEUCOCYTES

Third Experiment - Percent Incorporation and Degradation

	Incorporation			
	Percent of Control		Percent Depressed	
Substrate	0.1	0.5	0.1	0.5
Thymidine	55.1	25.8	44.9	74.2
Uridine	47.5	30.4	52.5	69.6
Leucine	87.8	57.9	12.2	42.1
Choline	168.2	138.2	-68.2	-38.2

	24-Hour Degradation: Percent of Zero Hour		
		Platinum	
Substrate	Control	0.1	0.5
Leucine	55.3	48.1	60.3
Choline	53.4	58.2	58.9

DISCUSSION

The animals were treated initially with varying doses of lead acetate and cadmium chloride to determine the highest level at which the animals would survive for the three-week experiment with daily i.p. injections. These levels were found to be 10 mg/kg for lead acetate and 1 mg/kg for cadmium chloride.

The lead and cadmium treatments produced a mild depression that lasted throughout the 7- and 21-day treatment periods. The weight losses seen in the control and experimental animals after both lead and cadmium treatments are thought to be in part a result of a lower food intake caused by the depressive effect of the metal treatments. Similar results were obtained with rats fed diets containing 300 ppm of lead in the form of lead acetate. Both weight loss and reduced food intake were noted [21].

Lead poisoning produces hemolytic, mild hypochromic, and sometimes microcytic anemia. Features include reticulocytosis which may be transitory, and basophilic stippling in the peripheral blood cells [22]. Stippling appears within 24 hours of exposure and may persist for 10 days to three weeks or more in rabbits [23]. The origin of lead-induced anemia is complex and not fully understood. Its course is profoundly affected by the level of, and route of exposure to, lead. Currently, it is believed that the anemia is primarily the result of shortened erythrocyte life span caused by mechanical fragility and reduced heme synthesis in red cell precursors [24] resulting from direct interference with synthesizing enzymes and possibly enzymes of glycolysis. Lead interacts with erythrocytes causing swelling, shrinkage, and crenation, which are typically seen in lead poisoning in man and animals, including rabbits. Crenation later disappears, and the attendant fragility leads to the shortened life span. The decrease in the red cell count is generally less than that of hemoglobin which seldom falls below 60%. The red cell count may diminish to $4 \times 10^6/mm^3$. Severe anemia is often accompanied by anisocytosis, poikilocytosis, and the appearance of Howell-Jolley bodies [23].

No consistent changes in white cell counts are observed, although leucocytosis, up to $15,000/mm^3$, is more commonly observed than leucopenia, especially in animals. Various effects have been noted on the differential count, including increases in the number of monocytes and lymphocytes. Eosinophilia has been noted by some workers but not by others. Granulocytosis that changes to a shift to the right as the lead poisoning becomes chronic has been observed. An *in vitro* exposure of leucocytes to lead chloride led to a loss of phagocytic ability [23].

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There is little in the literature concerning blood findings in cadmium poisoning. In workers in a battery factory the hemoglobin contents and red cell counts were normal or slightly low. The sedimentation rate was somewhat elevated. The white cell counts and differentials were normal with possible eosinophilia [25]. Tests on rabbits exposed to cadmium-iron dust or given daily subcutaneous injections of cadmium sulfate yielded similar results. Hemoglobins were slightly low, and the red cell counts were diminished from about $6.0 \times 10^6/mm^3$ to about $4.5 \times 10^6/mm^3$. White cell counts and differentials were normal with possible eosinophilia [25].

The results obtained in this research show that the three-week, but not the one-week, lead treatment produced a mild hemolytic anemia characterized by a diminished red cell count with the cells being larger than normal. The cadmium treatment produced an anemia characterized by a reduced blood hemoglobin content. Because the red cell count was normal and the hematocrit was low, it appears that the red cells were reduced in size as shown by the low MCV. However, the cells had a normal hemoglobin concentration as indicated by the MCHC value. These observations suggest an aberration in erythropoesis.

The effect of lead treatment on white cells was minor. White cell counts at both treatment periods were not significantly different from controls, and all values were within normal limits. The differential counts after both one and three weeks of lead treatment were nearly identical to the controls. Polychromasia and anisocytosis were more prevalent in the treated animals but were also seen in the control animals. It is interesting that basophilic stippling, a common characteristic of plumbism, was seen in only two of the lead-treated animals after one week of treatment and was not seen at all in the animals treated for three weeks.

In the one-week, cadmium-treated animals, the white cell count was elevated compared with that of the controls, but it was still within normal limits. After three weeks of cadmium treatment, the white cell count was again normal. The differentials showed a significant increase in neutrophils and a decrease in lymphocytes after one week of treatment. After three weeks, the neutrophil count became lower than that of the controls, but the difference was not significant. The lymphocyte count was normal. At both treatment periods, the appearance of aberrant cell forms was minor.

These observations for lead-treated animals are consistent with reports in the literature, especially in view of the reported variability of blood findings in plumbism, with the exception of anemia, stippling, and crenation. The anemia observed here in the cadmium-treated animals contrasts with Friberg's data [25] and may reflect differences in the conduct of the two experiments. In both cases, the apparent insensitivity of leucocyte counts and differentials to cadmium poisoning suggests that it is not a useful clinical parameter.

The biochemical analyses anticipated for the isolated leucocytes were abandoned when it was found that the dry weight associated with 2×10^6 cells was 0.9 mg. Thus it was impractical to attempt the separation of such a small mass into lipid, protein, RNA and DNA. It would have been possible to add a known amount of a carrier, such as yeast cells, to each sample to ensure the recovery of each fraction, but since the purpose of isolating these fractions was to determine the specific activity of the particular radioactive precursor in each fraction, the addition of a carrier would preclude a mass determination.

For determining the radioactivity content of the cells, isotopically labeled substrates were combined in ^{14}C-^{3}H pairs for incubations. The Searle Mark III LSC is capable of resolving the two types of radioactive disintegrations. In all cases, the combination of washing labeled cells with a 100-fold excess of unlabeled substrate and the subsequent TCA precipitation ensures that unbound labeled precursors are eliminated in the acid-soluble fraction from the cells.

During the 22-hour incubation in the presence of radioactive precursors, it is reasonable to expect some redistribution of label by metabolism of the radioactive substrates. Thus, for example, the inference that cellular tritium derived from ^{3}H-leucine represents protein is not entirely correct. However, the incorporation of radioactive substrates in heavy metal-treated cells is always compared with that of control cells, so that for the purpose of detecting altered rates of metabolism due to heavy metal treatment, the procedure is adequate.

The incorporation of radioactivity observed in these experiments is due to uptake by the leucocytes and not due to other potential sources of metabolic activity. For example, bacterial contamination has never been observed. Aliquots of blood samples were cultured on tryptic soy agar plates and no bacterial growth was observed. Although this single test does not ensure that more fastidious organisms are not present, it is indicative of the absence of gross bacterial contamination. Mansfield reports (private communication) that pleuropneumonia-like organisms (PPLO) are a frequent accompaniment of rabbit blood cell cultures, but that their growth is effectively suppressed by the inclusion of tylosin in the culture medium. This precaution was routinely observed.

Blood platelets are metabolically active [26], but these cells are largely removed during two steps in the preparation of the leucocyte suspension: removal of the platelet-rich plasma and washing of the final leucocyte suspension.

As indicated earlier, contamination of the leucocyte suspension by red blood cells was unavoidable. The RBC/WBC ratio rarely fell below 1 and ranged up to 8 (Tables 25 and 26). Leucocyte preparations with a greater red cell contamination were not used except that from the #6 lead-treated animal in the three-week experiment, which had a ratio of 37.5. The RBC control experiment clearly demonstrates that, in general, the contribution of radioactivity incorporation due to RBC is very much

less than 10%. However, in two of the animals the WBC incorporations were small enough that the potential contribution due to RBC invalidates some of the data. The table below shows the percent of the WBC incorporations that may be attributable to the RBC content for several of the WBC preparations having the lowest cell count combined with the highest RBC contamination.

	Experiment (weeks)				
	1	1	1	3	3
Treatment:	Lead	Lead	Cadmium	Cadmium	Lead
Animal:	#1	#5	#5	#5	#6
RBC/WBC:	8.2	5.4	6.7	1.6	37.5
Thymidine (%)	1.8	6.6	47	6.9	100
Uridine (%)	0.3	2.9	3.0	1.4	6.2
Leucine (%)	3.3	-	10	2.1	47
Choline (%)	1.7	-	2.7	0.2	16

The presence of red cells in the leucocyte preparations seems undesirable but, at the present time, inescapable. As indicated earlier, the one procedure attempted for the hypotonic lysis of RBC irreparably damaged the leucocytes as well. However, this procedure was designed for human cells. Mansfield (personal communication) reports that another procedure [27] for hypotonic lysis worked well with rabbit leucocyte preparations. We have found that this method completely eliminates red blood cells from the leucocute suspensions, and we are currently evaluating the integrity of the resultant white cells in preparation for future research.

The low incorporation of radioactive substrates into the red blood cells is consistent with the known metabolism of these highly specialized cells [28,29]. For example, since erythrocytes have no nucleus, they cannot biosynthesize DNA or RNA. Thus, the incorporation of labeled thymidine and uridine into a mixed population of red and white cells should be due primarily to the white cells, particularly in the circumstance when DNA synthesis and also, therefore, RNA and protein synthesis have been stimulated by the mitogen, phytohemagglutinin. Some incorporation may be due to the small proportion of reticulocytes as well. Also, mature red cells do not synthesize lipids or protein. There is, however, an energy-independent exchange of cholesterol and, to a lesser extent, phospholipid between plasma and the red cell membrane.

Similarly, in dogs and humans, the rate of glycolysis in erythrocytes was 0.014 to 0.028 μg glucose/hr/10^6 cells, whereas in leucocytes the rate was 4.7 to 13.2 μg glucose/hr/10^6 cells [29]. Thus, the glyolytic rate in erythrocytes was, at most, 0.6% of the rate found in leucocytes. Only a few of the tricarboxylic acid enzymes persist in the adult erythrocyte. It seems likely that the situation would be similar in rat and rabbit cells.

The greatest difficulty encountered in this research was associated with the separation of white cells from rabbit blood and maintaining their viability during the subsequent culturing. The major problems were low white cell yields, white cell clumping or aggregation, high red cell contamination, and short viability of the white cells. Repeated efforts were made to improve these aspects of the procedure, using many techniques that the literature indicated as being satisfactory for human cells; however, all of them failed to produce acceptable rabbit white cell preparations. Successful preparations were not obtained until the paper of Mansfield and Wallace [14] appeared and we adopted their procedures. Several important factors were indicated in their paper and in direct communication with them: the use of heparin (and not EDTA) as an anticoagulant; the use of up to 100 U/ml of heparin to prevent platelet aggregation and RBC agglutination; the use of RPMI-1640 culture medium; the inclusion in the medium of heat-inactivated autologous plasma as the protein supplement; the inclusion in the medium of tylosin (at a level of 10%) as an anti-PPLO agent; the use of 1×10^6 white blood cells per milliliter of culture medium; the use of low levels of PHA to stimulate mitogenesis; and extension of the PHA treatment time to 22 hours prior to addition of the radioactive substrates. Other factors that we found to be important, and in which Mansfield concurred (private communication), are the dilution of whole blood 1:2.5 with HBSS, and centrifugation on the gradient for at least 30 minutes and at 400 x g or more.

Because of the repeated inability to obtain a good leucocyte preparation, we investigated numerous other variables, none of which had a significant enough effect on the procedure to warrant a change. Among these are: obtaining the blood by opening the chest cavity before cardiac puncture; pretreating the rabbit with heparin prior to obtaining blood by cardiac puncture; the heparin concentration within the limits of 10-100 U/ml; the age of the blood up to about six hours after drawing; filtering the blood through sterile cheesecloth or glass wool to remove small clots of red cells; the freshness of the Ficoll and Hypaque solutions (they are mixed just prior to use); autoclaving Ficoll but not the Ficoll-Hypaque mixture; layering the gradient beneath the blood as opposed to layering the blood on top of the gradient; and increasing the time or speed of centrifugation by up to 50%.

The incorporation of labeled thymidine, uridine, leucine, and choline by viable leucocytes implies the biosynthesis of DNA, RNA, protein, and phospholipid, respectively. The problem of the redistribution of label during the incubation period was discussed earlier.

The substrates employed are among the more metabolically stable biochemicals found in living matter and are frequently used to monitor the above biosynthetic processes.

The data clearly show that leucocytes demonstrated to be viable by the eosin Y test are also very active metabolically in synthesizing the major classes of biochemical constituents of nucleic acids, protein, and

lipid. Furthermore, the leucocyte preparations exhibit the dynamic nature of cellular metabolism characteristic of living cells in that the labeled protein and phospholipid in the leucocytes undergo degradation, thus implying a continual renewal of these materials. These observations serve to demonstrate that the leucocyte preparations are healthy--or at least sufficiently so to serve as monitors for examining the metabolic effects of heavy metal treatment in the host animals from which the leucocytes are derived.

The data from the preliminary lead and cadmium experiment indicate that lead and cadmium treatments of rabbits have important metabolic effects. The predominant effect noted is a severe depression in the biosynthesis of DNA in the cadmium-treated animal. This observation was confirmed in the one-week and three-week experiments. The greatest depression was seen after one week of cadmium treatment. By three weeks, DNA synthesis was still reduced but less so than at one week.

RNA synthesis is also affected by cadmium, but the depression is much less than with DNA. The depressive effect of cadmium on DNA synthesis was about the same at one and three weeks.

In contrast to these results, lead treatment in the preliminary experiment and in the one-week experiment had negligible effect on either DNA or RNA synthesis. By three weeks, nucleic acid synthesis was suppressed but less so than that due to cadmium exposure.

Protein and phospholipid biosyntheses were not affected by either lead or cadmium treatment for one or three weeks. In the preliminary experiment, protein biosynthesis was not affected by the lead treatment, but was decreased by cadmium exposure. In contrast, phospholipid synthesis appeared to be accelerated by both treatments. Since this result was not repeated in the one- and three-week experiments in which more animals were used, we assume that the phospholipid results from the preliminary experiment are probably not representative.

In general, protein and phospholipid degradations were not affected by lead or cadmium treatment in the one- or three-week experiments. Two exceptions were noted in that phospholipid turnover was increased by lead treatment at 24 hours and by cadmium treatment at 5 hours (Table 41). The increases were small but statistically significant. The preliminary experiment gave the same result for the lead treatment but indicated that both protein and phospholipid turnover were increased by cadmium treatment. Again, this result is not considered representative.

The effect of cadmium and lead treatment <u>in vivo</u> on the metabolism of leucocytes does not appear to have been studied previously [30 91-(1972), although extensive work has been carried out in other tissues from many species. Lead and cadmium bind to a wide variety of biomolecules by virtue of their strong affinity for purine, pteridine, porphyrin, and phosphate moieties and for sulfhydryl, histidinyl, phenoxy, and

side-chain amino acid carboxyl groups of proteins. Lead and cadmium inhibit many enzymes having functional sulfhydryl groups, particularly those associated with oxidative phosphorylation and, hence, synthesis [31] and with nucleic acids. On the other hand, they can either stimulate or inhibit several peptidases by substituting or interacting with resident zinc atoms [30].

In this research, inhibition of the incorporation of thymidine into nucleic acids by cadmium and lead treatment was the outstanding metabolic feature. The incorporation of leucine into protein, of choline into phospholipid, and of uridine into ribonucleic acid is energy-dependent requiring the participation of ATP. Because these processes were not affected by the lead and cadmium treatments, or only minimally so, it may be deduced that energy metabolism and oxidative phosphorylation were not appreciably affected.

Since the incorporation of thymidine into deoxynucleic acid is also energy-dependent, it is reasonable to infer that the lead and cadmium treatments interfered with DNA synthesis in a manner not involving energy metabolism. Cadmium and other metals affect the physical properties of DNA, but their biological significance has not been explored [32]. Lead forms complexes of nucleotides and nucleic acids through the phosphate group [33]. Lead concentrates in the cell nuclei of liver and kidney [34,35] and probably also in leucocytes as judged by the appearance of chromosomal abnormalities in human [36] and mouse white cells [37]. Thus, it is possible that the depressive effect of the cadmium and lead treatments on DNA synthesis may be a result of a direct interaction of these metals with DNA which subsequently disrupts the replication process. Further research would be required to investigate this hypothesis.

Thymidine incorporation occurs at varying rates in leucocytes according to cell type and to the biochemical and physiological status of each cell type. This incorporation depends on such factors as the size of the purine and pyrimidine pools, the number of mitotically active cells at any given time, the extent to which labeled thymidine is diverted to non-DNA pathways, and the extent to which the labeled moieties of DNA are reutilized [38]. The extent to which such factors influenced the results of this research cannot be assessed, but it seems pertinent to observe that the experiments were conducted as identically as possible, with the exception of the treatment regimen, in order to minimize procedural variations. Thus, it seems reasonable to suggest that the differences in incorporation of labeled thymidine is attributable to the metal treatment which, in ways that are obscure at present, altered one or more factors controlling the incorporation of labeled thymidine. Because the shifts in the white cell differentials caused by metal treatment were small, it may be concluded that a shift in cell type is not responsible for the variations in incorporation.

A final observation concerning the one- and three-week experiments is that the level of incorporation of all four radioactive substrates was significantly higher in the three-week control animals than in the

one-week controls (Table 42, section B). The animals for the two experiments were purchased together and randomly placed on the one- or three-week treatment regimes; hence, variations due to age and sex can be eliminated. There was no correlation between incorporation of radioactive substrates and the sex of the rabbits. To obtain sufficient blood for the leucocyte preparation, adult rabbits having a minimum weight of 3 kg were specified (three rabbits weighing less were used--see Tables 19 and 20). Because of differing growth rates, the ages of the animals used also varied. Although data on ages were not presented, there was no correlation between incorporation of radioactive substrates and the age of the rabbits. Animal handling, care, and treatment were identical for both groups of animals except for the duration of treatment. The procedures for obtaining the blood and for white cell preparation and use were conducted in as identical a manner as possible to avoid any biochemical effects that might be caused by procedural variations.

The primary difference between the one- and three-week controls was that the latter incurred an additional two weeks of stress caused by daily i.p. injections of a saline control. Biochemical alterations in animals are well-known concomitants of stress and include release of ACTH through activation of the adrenal-pituitary axis [39] and alterations in microsomal drug-metabolizing enzymes [40]. In most studies, the stress appeared to be more extreme than that caused by daily i.p. injections of saline. However, subtle alterations of leucocyte metabolism may represent a sensitive response to stress not previously recognized.

Unexpectedly, the results of the three in vitro platinum experiments lacked consistency. The third experiment was performed in the most favorable manner in that both the control and platinum-treated cells were washed twice in saline to maximize removal of platinum salt from the treated cells. In the first two experiments, because the two wash steps were omitted, it is possible that the leucocytes might have been more numerous and more vigorous than those in the treated suspensions. The results of the second experiment seem less satisfactory than the other two in that (a) there is little variation in the percent depression of the various labels, and (b) the depression of incorporation is higher for the lower treatment level.

One point of constancy regarding the first and second experiments is in the absolute incorporations of the four labeled substrates in the control cells compared to the corresponding incorporations in the control cells from the one- and three-week experiments. This is expected, of course, since the same pool of leucocytes was used in both cases. While the first and third experiments vary in the magnitude of the various depressions of incorporation, there is a constancy in that platinum treatment of 0.5 mg/ml depresses thymidine incorporation to the greatest degree, uridine second, and leucine third. Choline uptake is the least affected in both experiments, with only a slight depression in the first experiment and an apparent stimulation in the third.

Considering all three experiments together, it seems apparent that platinum treatment at 0.5 mg/ml does interfere with cellular metabolism and in particular with nucleic acid synthesis. Protein synthesis is also probably affected, while the effect on phospholipid metabolism is small. It also seems evident that platinum affects nucleic acid metabolism at the lowest level tested, 0.1 mg/ml.

Although these three in vitro experiments show considerable variation in results, this type of experiment has a major advantage over the type of experiments performed in the one- and three-week exposures. In the in vitro experiments, the treated cells and the control cells are derived from the same pool, whereas in the one- and three-week experiments the control cells and the treated cells are derived from different animals. This is, of course, a necessary concomitant to the study of in vivo effects of heavy metal treatment, but it does necessitate reliance on a statistical evaluation of results from several animals. We believe that such an analysis has been successfully accomplished, particularly through using the tactic of comparing the uridine/thymidine and choline/leucine ratios, which normalize the data and eliminate much of the inherent biological variation among a small population of animals.

Determining the extent of recent and past exposure to trace metals is difficult. Blood and urine determinations are used as indices of recent and current exposure to lead, but they are inadequate in assessing previous exposure because of its storage in kidney, liver, bone, and other tissues. Thus, the blood level may be normal or minimally elevated, but the body burden may be very high. Determinations of lead in hair gives information on previous exposures but not on present metabolic status. One method currently used to estimate body burden involves mobilization of tissue stores of lead using CaEDTA and measurement of urinary excretion of the lead complex.

Cadmium is also stored in tissues, particularly in the liver and kidney. Thus, levels of blood cadmium, like blood lead, may not reflect the degree of intoxication. Also cadmium analysis presents such difficulties as tissue preparation, interference from much larger amounts of zinc, and sensitivity of the assay. More recent methods, such as flameless atomic absorption analysis using a carbon rod atomizer (Varian Techtron), should facilitate such analyses. In both cases, tissue analysis would be desirable, but acquisition of tissue samples is generally not feasible. Thus, other methods for the determination of body burden of lead and cadmium would be useful.

The work described here demonstrates that the white blood cell is a responsive bioindicator of lead and cadmium intoxication in the rabbit. Further work is required to relate both the body burden of these metals to the degree of leucocyte response and the sensitivity of the response to metal intake.

The leucocyte has several attributes that are ideal in a bioindicator for trace metal contamination. It is a complete cell possessing a nucleus,

mitochondria, and microsomes in contrast, for example, to the mature red blood cell (erythrocyte) which lacks these organelles. Further, normal leucocytes are important in many of the body's defense mechanisms, and the appearance of abnormal forms is often a manifestation of significant disease processes. Thus, the leucocyte may be expected to possess a diversity of biochemical processes that equal or exceed those of other nucleated and differentiated cells of the body. Finally, leucocytes are a readily available biopsy tissue that can be separated from other blood cells in relatively pure form for subsequent studies of morphology, biochemistry, physiology, and immunochemistry. It is anticipated that the use of leucocytes as bioindicators will be expanded to include a variety of compounds other than trace metals and extended in relevancy by the use of human leucocytes.

SUMMARY

1. Rabbit leucocytes were successfully separated from whole blood and cultured, using the procedure of Mansfield and Wallace [14]. The essential features of this procedure are separation of white cells from heparinized whole blood by density gradient centrifugation on a Ficoll-Hypaque solution and culture of white cells in RPMI-1640 medium containing 10% heat-inactivated autologous rabbit plasma.

2. The metabolism of the white cells was stimulated by a 22-hour treatment with the plant mitogen, phytohemagglutinin (PHA).

3. Untreated, viable control cells were shown to synthesize DNA, RNA, protein and phospholipid by the incorporation of ^{3}H-thymidine, ^{14}C-uridine, ^{3}H-leucine, and ^{14}C-choline, respectively. In addition, protein and phospholipid were shown to catabolize in previously labeled cells following the removal of labeled leucine and choline, respectively, from the culture medium.

4. The intraperitoneal injection of rabbits *in vivo* with lead acetate or cadmium chloride solutions for one or three weeks resulted in well-characterized alterations in the metabolism of leucocytes derived from the treated rabbit.

5. The most outstanding effect noted was a severe depression in nucleic acid synthesis, particularly DNA, caused by cadmium treatment for one or three weeks or by lead treatment for three weeks. Lead treatment for one week had no influence on nucleic acid synthesis.

6. Lead and cadmium treatment had little or no effect on the biosynthesis or degradation of protein or phospholipid after either one or three weeks of treatment.

7. Leucocytes from three-week control rabbits, which received daily i.p. injections of saline, incorporated 402% more thymidine, 134% more uridine, 88% more leucine, and 68% more choline than did leucocytes from the one-week control rabbits. It is suggested that the extra two weeks of stress caused by the injections was responsible for the increased incorporations.

8. The one- and three-week control and metal treatments produced a mild weight loss in all animals. At the end of one week of treatment, the weight loss in the metal-treated animals was significantly greater than that in the controls, with the lead-treated animals exhibiting the greatest loss. After three weeks, the weight loss of the lead-treated animals, but not of the cadmium-treated animals, was significantly greater than that of the controls. These losses are thought to result from reduced food intake.

9. The one-week lead and cadmium treatments had no effect on red cell findings. The three-week lead treatment produced a mild hemolytic anemia characterized by a diminished red cell count, hemoglobin content, and hematocrit and an increased red cell size. Anemia occurred after the three-week cadmium treatment as a result of a low hemoglobin content and small red cells with a normal hemoglobin concentration.

10. After one week of cadmium treatment, the white cell count was significantly elevated, but by three weeks it had returned to normal. Neither the one-week nor the three-week lead treatments had an effect on the white cell count.

11. Lead treatment had no effect on the white cell differentials. Polychromasia and anisocytosis were more prevalent after one week of lead treatment and were normal by three weeks. Basophilic stippling, a characteristic of chronic lead poisoning, was observed in only two animals and only after one week of treatment. Cadmium treatment produced an elevated neutrophil count and reduced lymphocyte count at one week. After three weeks, the neutrophil count was low, and the lymphocyte count was normal. Aberrant cell forms were infrequently seen in the cadmium-treated animals.

12. When control leucocytes were treated in vitro with sodium hexachloroplatinate for two hours, synthesis of nucleic acid, particularly DNA, is depressed. This effect is noted at the lowest salt concentration used, 0.1 mg/ml of culture medium. Protein synthesis is also depressed, but phospholipid synthesis is only slightly affected.

13. In one experiment, degradation of protein and phospholipid was not affected by platinum treatment even at the lowest level used, whereas in another experiment the degradation rate was slowed by a factor of two. Thus, the effect of platinum treatment on the degradation of protein and phospholipid is uncertain.

GLOSSARY

Cd, CD	Cadmium
cpm, CPM	Counts per minute
DNA	Deoxyribonucleic acid
DF	Degrees of freedom (see "v")
dpm, DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
Hb	Hemoglobin
HBSS	Hank's balanced salts solution
Hp	Hypaque
LD_{50}	Median lethal dose, one that is fatal to 50% of the test animals
MC	Methylcellulose
MCH	Mean corpuscular hemoglobin--the hemoglobin content of the average red blood cell
MCHC	Mean corpuscular hemoglobin concentration--the hemoglobin concentration in the average red blood cell
MCV	Mean corpuscular volume--the volume of the average red blood cell
MEM	Eagle's minimal medium
MEM-S	Eagle's minimal medium, spinner modification
μc	Microcurie = 10^{-6} curie = 2.22×10^{6} dpm
NSC™ Solubilizer	A solution of a quaternary ammonium base in toluene developed by Amersham/Searle for the solubilization of biological materials
NRBC	Nucleated red blood cells

P	The probability that the two samples examined are statistically significantly different from each other. P > 95% is considered significant.
Pb, PB	Lead
PHA	Phytohemagglutinin--this material is available from Difco as either the M or the P form, the latter having the highest purity
Pt	Platinum
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI-1640	A nutrient medium for tissue culture devised at the Roswell Park Memorial Institute, New York
s	A statistical parameter relating to Kendall's coefficient of concordance
SD	Standard deviation
t, T	The t-value from the Students' t-Test
U	Units
v	Degrees of freedom, a statistical parameter employed in the Students' t-Test
W	Kendall's coefficient of concordance
WBC	White blood cells

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